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Novel Posttranslational Modification in LKB1 Activation and Function

Szu-Wei Lee

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NOVEL POSTTRANSLATIONAL MODIFICATION IN LKB1 ACTIVATION AND FUNCTION

by

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Novel Posttranslational Modification in LKB1 Activation and Function

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Szu-Wei Lee, M.S.

Houston, Texas

August, 2014

Dedication

This Ph.D. thesis work is dedicated to

My dearest parents

Acknowledgements

First of all, I would like to thank my parents, who live in Taiwan, for their unconditional love and unqualified support in my life. Without them, I could not have made it through and become who I am now. Here I would like to express my deepest appreciation to my dearest parents for all that they have done for me.

Secondly, I am very thankful to my mentor—Dr. Hui-Kuan Lin—for his guidance and support over the years. He has provided an outstanding scientific environment for me to learn and do research. Being in his lab, I have had a great chance to know the real science. Also, I sincerely appreciate having professors, Drs. Dos Sarbassov, Jianping Jin, Jessica Tyler, Xin Lin, Elsa Flores, Jian Kuang and Mong-Hong Lee on my committees during the course of my Ph.D. training. Their professional, experienced opinions and suggestions have been very constructive. I would particularly like to extend my respect and regards to Dr. Sarbassov, who is just next door to me, for his care and advice about my career.

Next, I would like to take this opportunity to thank all the current and past lab members for their intellectual input, cooperation and friendship. Especially, I am extremely grateful to Drs. Chia-Hsin (Lori) Chan, Wei-Lei (William) Yang, Jing Wang and Guoxiang Jin, and Yuan Gao for their kind help and support in both my research and life. Others include Fei Han, Xian Zhang, Yun-Seong Jeong, and Drs. Hossein Rezaeian and Zhen Cai (there are too many others to list). They all are very nice and helpful to me, and I appreciate working with any of them. In addition, I would like to thank our collaborator Dr. Chien-Feng Li (Chi-Mei Medical Center, Taiwan) for his technical help and support in my research project.

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Novel Posttranslational Modification in LKB1 Activation and Function

Szu-Wei Lee, M.S.

Supervisory Professor: Hui-Kuan Lin, Ph. D.

Cancer cells display dramatic alterations in cellular metabolism to meet their needs of increased growth and proliferation. In the last decade, cancer research has brought these pathways into focus, and one emerging issue that has come to attention is that many oncogenes and tumor-suppressors are intimately linked to metabolic regulation (Jones and Thompson, 2009). One of the key tumor-suppressors involved in metabolism is Liver Kinase B1 (LKB1). LKB1 is the major upstream kinase of the evolutionarily conserved metabolic sensor—AMP-activated protein kinase (AMPK). Activation of the LKB1/AMPK pathway provides a survival advantage for cells under energy stress.

LKB1 forms a heterotrimeric complex and is activated through binding of the two regulatory proteins, STRAD and MO25. LKB1 has been shown to be a tumor-suppressor in various mouse models; however, recent studies suggest that LKB1 has pro-oncogenic functions. How the LKB1 activity and the LKB1-STRAD-MO25 complex are maintained and regulated and how LKB1 regulates cancer development are largely unclear. Here we show that K63-linked LKB1 polyubiquitination by the Skp1-Cul1-F-box-protein/Skp2 (Skp2-SCF) ubiquitin ligase complex is critical for LKB1 activation by a mechanism of maintaining the LKB1-STRAD-MO25 complex integrity. We further demonstrate that oncogenic Ras acts upstream of Skp2 to promote

LKB1 polyubiquitination by activating the Skp2-SCF ubiquitin ligase complex. Moreover, Skp2-mediated LKB1 polyubiquitination is required for energy stress-induced cell survival. We also detected upregulation and positive correlation of Skp2 and LKB1 expression in late-stage hepatocellular carcinoma (HCC), and their overexpression predicts poor survival outcome of HCC patients. Finally, we show that Skp2-mediated LKB1 polyubiquitination is important for HCC tumor growth in a mouse subcutaneous xenograft tumor model. Our study provides new insights into the upstream regulation of LKB1 activation and suggests a potential target, the Ras/Skp2/LKB1 axis, for cancer therapy.

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LIST OF ABBREVIATIONS

| | |
|---------------|--|
| 2-DG | 2-deoxyglucose |
| ACC | Acetyl Co-A carboxylase |
| AICAR | 5-aminoimidazole-4-carboxamide ribonucleotide |
| AMPK | AMP-activated protein kinase |
| ARK | AMPK-related kinase |
| BRSK | Brain-specific kinase |
| CAMKK β | Calmodulin-dependent protein kinase kinase β |
| Cul1 | Cullin 1 |
| DMEM | Dulbecco's Modified Eagle's medium |
| E | Embryonic day |
| EGF | Epidermal growth factor |
| Erk | Extracellular signal-regulated kinase |
| FBS | Fetal bovine serum |
| FBXL | F-box protein with leucine-rich repeats |
| FBXO | F-box protein with other diverse domains |
| FBXW | F-box protein with WD40 domains |
| Grr1 | Glucose repression-resistant 1 |
| GST | Glutathione S-transferase |
| HECT | Homologous to E6-associated protein C-terminus |
| H-Ras | Harvey rat sarcoma viral oncogene homolog |
| IGF | Insulin-like growth factor 1 |
| KD | Kinase-dead |
| KO | Knockout |
| LKB1 | Live Kinase B1 |
| LOH | Loss of heterozygosity |

| | |
|----------|---|
| MAPK | Mitogen-activated protein kinase |
| MARK | Microtubule affinity-regulating kinase |
| MEK | MAPK/Erk kinase |
| MEF | Mouse embryonic fibroblast |
| MO25 | Mouse protein 25 |
| mTOR | Mammalian target of rapamycin |
| p90Rsk | 90-kDa ribosomal S6 kinase |
| PCR | Polymerase chain reaction |
| PJS | Peutz-Jeghers syndrome |
| Raptor | Regulatory associated protein of mTOR |
| RING | Really interesting new gene |
| SCF | Skp1-Cul1-F-box-protein |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| shRNA | Short hairpin RNA |
| SIK | Salt-inducible kinase |
| Skp1 | S-phase kinase-associated protein 1 |
| Skp2 | S-phase kinase-associated protein 2 |
| SNF1 | Sucrose non-fermenting 1 |
| STARD | Ste20-related adaptor |
| STK11 | Serine/threonine kinase 11 |
| TSC | Tuberous sclerosis |
| UBC | Ubiquitin-conjugating enzyme |
| UBD | Ubiquitin-binding domain |
| ULK1 | Unc-51-like kinase 1 |
| WT | Wild-type |

Chapter 1

Introduction

1.1. *LKB1* as a Tumor-suppressor in Human

LKB1 (liver kinase B1) (also known as *STK11*; serine/threonine kinase 11) was first identified as a tumor-suppressor gene, because its germline mutations have been linked to the cancer-prone Peutz-Jeghers syndrome (PJS), an autosomal dominant inherited disorder (Hemminki et al., 1998; Jenne et al., 1998). The main characteristic of this syndrome is the development of benign hamartomatous polyps in the gastrointestinal tract and hyperpigmented patches in the mouth. PJS patients have an increased risk of developing cancers in various organs, particularly gastrointestinal tumors (Giardiello et al., 2000; Sanchez-Cespedes, 2007), and about 80% of them were found to carry a *LKB1* mutation (Volikos et al., 2006). Later on, somatic alterations of *LKB1* were identified in sporadic cancers; however, the overall incidence is typically rare (Sanchez-Cespedes, 2007). Exceptionally, *LKB1* gene inactivation is common in lung and cervical cancers. *LKB1* is frequently mutated in ~20% of cervical cancer (Wingo et al., 2009). On the other hand, the frequency of *LKB1* mutations is ~30% (ranging from 10 to 50%) in non-small cell lung cancer, and it is most prevalent in lung adenocarcinoma (Sanchez-Cespedes, 2007; Sanchez-Cespedes, 2011). Noteworthy, *LKB1* mutations were found frequently accompanied by alterations in other genes, such as *TP53*, *P16*, and especially *KRAS* in smoking non-Asian patients, but rarely concomitant with mutations in *EGFR* (Sanchez-Cespedes, 2011), which are highly associated with non-smoker Asian patient population (El-Telbany and Ma, 2012; Kadara et al., 2012). Loss of *LKB1* protein expression was observed in high-grade atypical adenomatous hyperplasia, a preneoplastic lesion for lung adenocarcinoma (Ghaffar et al., 2003), suggesting a role of *LKB1* inactivation in the early development of this type of cancer. Nonetheless, the initiating event has been suggested to be the activating mutations of *KRAS* (Westra, 2000). In *Kras* lung cancer mouse model, loss of *Lkb1* promotes lung cancer initiation and metastasis (Ji et al., 2007).

1.2. *Lkb1* Genetic Mouse Models

Lkb1 knockout (KO) mice die before embryonic day (E) 11 (at about E8.5-11), which are embryonic lethal, with developmental defects in neural tube closure and vascularization (Ylikorkala et al., 2001). *Lkb1* heterozygous KO mice were shown to develop gastrointestinal polyps which resemble PJS polyps (93% in stomach and 31% in small intestines at >20 and >50 weeks of age, respectively) (Miyoshi et al., 2002), and also develop different types of tumors with a relatively long latency reported in different studies, such as benign osteogenic tumor at ~43 weeks of age (Robinson et al., 2008), invasive endometrial adenocarcinoma by 55 weeks of age (Contreras et al., 2008), and hepatocellular carcinoma (HCC) at >50 weeks of age (Nakau et al., 2002), concurrent with or without loss of wild-type (WT) *Lkb1* allele (loss of heterozygosity; LOH) (Table 1-1). Mice with conditional inactivation of *Lkb1* in various types of cells or tissues through homologous recombination by cell- or tissue-specific expression of Cre recombinase showed either tumorigenic or non-tumorigenic phenotypes, suggesting the context- or tissue-specific functions of *Lkb1* in tumor suppression and the critical roles of *Lkb1* in development of specific tissues or organs (Ollila and Makela, 2011; Shorning and Clarke, 2011) (Tables 1-1 and 1-2). The important roles of *Lkb1* in embryogenesis, angiogenesis, and development of nervous system, hematopoietic system, pancreas, muscle and liver have been demonstrated in the mouse models (Table 1-2). Loss of *Lkb1* in combination with other tumorigenic mutations (i.e., activated oncogenes and inactivated tumor-suppressor genes) results in promoting tumorigenesis and accelerating tumor development in multiple tissues (Ollila and Makela, 2011) (Table 1-1). This suggests the existence of genetic interactions between *Lkb1* and other genes important for tumorigenesis, which adds complexity to deciphering the role of *Lkb1* in tumor development.

Table 1-1. *Lkb1* mouse models with tumorigenic phenotypes

| Genotype | Targeting | Phenotypes | Latency (wks)* | LOH or H | References |
|---|--------------------------------------|---|--------------------------------------|----------|--|
| <i>Lkb1</i> ^{+/-} | All tissues | Gastric hamartomas | 21-30 (67%) | H | (Miyoshi et al., 2002) |
| <i>Lkb1</i> ^{+/-} | All tissues | Gastric hamartomas | >24 (100%) | H | (Rossi et al., 2002) |
| <i>Lkb1</i> ^{+/-} | All tissues | Gastric hamartomas | 40-56 (100%) | H | (Jishage et al., 2002) |
| <i>Lkb1</i> ^{+/-} | All tissues | Gastrointestinal hamartomas | <43 (68%) | LOH | (Bardeesy et al., 2002) |
| <i>Lkb1</i> ^{+/-} | All tissues | Osteogenic tumor | ~43 | H | (Robinson et al., 2008) |
| <i>Lkb1</i> ^{+/-} | All tissues | Hepatocellular carcinoma | >50 (71%) | LOH | (Nakau et al., 2002) |
| <i>Lkb1</i> ^{+/-} | All tissues | Endometrial adenocarcinoma | <55 (53%) | H | (Contreras et al., 2008) |
| <i>Lkb1</i> ^{flox/flox} ; Adeno-Cre (i.u.) | Uterine cells | Endometrial adenocarcinoma | <36 (65%) | n/d | (Contreras et al., 2008) |
| <i>Lkb1</i> ^{+/-} | All tissues | Carcinogen-induced squamous cell carcinoma | 15 | LOH | (Gurumurthy et al., 2008) |
| <i>Lkb1</i> ^{flox/flox} ; Keratin-14-Cre | Epidermal cells | Carcinogen-induced squamous cell carcinoma | 8 (100%) | n/d | (Gurumurthy et al., 2008) |
| <i>Lkb1</i> ^{+/-flox} ; Tagln-CreERT2 (i.p., 4-OHT) | Gastrointestinal smooth muscle cells | Gastrointestinal hamartomas | 45 (61%) (75% for <i>flox/flox</i>) | H | (Katajisto et al., 2008) |
| <i>Lkb1</i> ^{flox/flox} ; BLG-Cre | Mammary epithelial cells | Ductal carcinomas or solid papillary carcinomas | 46-85 (19%) | n/d | (McCarthy et al., 2009) |
| <i>Lkb1</i> ^{flox/flox} ; Pdx1-Cre | Pancreatic epithelial cells | Pancreatic serous cystadenomas | 10-30 (100%) | n/d | (Hezel et al., 2008) |
| <i>Lkb1</i> ^{flox/flox} ; Ah-Cre | Prostate epithelial cells | Prostate intraepithelial neoplasia | 8 (83%) | n/d | (Pearson et al., 2008) |
| <i>Lkb1</i> ^{flox/flox} ; Sprr2f-Cre | Uterine epithelial cells | Invasive endometrial cancer | 12 | n/d | (Contreras et al., 2010) |
| <i>Lkb1</i> ^{+/-} | All tissues | Gastric hamartomas | 26 (75%) | n/a | (Wei et al., 2005) |
| <i>Lkb1</i> ^{+/-} ; <i>Tp53</i> ^{-/-} | All tissues | Accelerated gastric hamartomas | ~16 (88%) | n/a | (Wei et al., 2005) |
| <i>Lkb1</i> ^{+/-} | All tissues | Gastric hamartomas | >20 (33%) | n/d | (Takeda et al., 2006) |
| <i>Lkb1</i> ^{+/-} ; <i>Tp53</i> ^{-/-} | All tissues | Accelerated gastric hamartomas | 12 (100%) | No LOH | (Takeda et al., 2006) |
| <i>Lkb1</i> ^{+/-} ; <i>Tp53</i> ^{-/-} | All tissues | Accelerated hepatocellular carcinoma | 25 (8%) | LOH | (Takeda et al., 2006) |
| <i>Lkb1</i> ^{+/-hypo} ; <i>Pten</i> ^{+/-} | All tissues | Large B-cell follicular lymphomas | 24-32 (80%) | n/d | (Garcia-Martinez et al., 2011; Huang et al., 2008) |
| <i>Lkb1</i> ^{+/-} ; <i>Catnb</i> ^{+/-lox(ex3)} ; Adeno-Cre (i.v.) | All tissues | Accelerated hepatocellular carcinoma | n/a | LOH | (Miyoshi et al., 2009) |

Table 1-1. *Lkb1* mouse models with tumorigenic phenotypes (continued)

| Genotype | Targeting | Phenotypes | Latency (wks)* | LOH or H | References |
|---|-----------------------------|-------------------------------------|-----------------|----------|-----------------------------|
| <i>Lkb1</i> ^{flox/flox} ; <i>Pten</i> ^{flox/flox} ; <i>Ah-CreER</i> TM (i.p., β -NF, tam) | Bladder epithelial cells | Papillary bladder tumors | <18 | n/d | (Shorning et al., 2011) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Pten</i> ^{flox/flox} ; Adeno-Cre (i.u.) | Uterine cells | Endometrial tumors | 8-28 (100%) | n/d | (Cheng et al., 2014) |
| <i>Lkb1</i> ^{flox/flox} ; <i>MMTV-ErbB2-Cre</i> | Mammary epithelial cells | Mammary gland tumors | 17-18 | n/d | (Dupuy et al., 2013) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Misr2-Cre</i> | Gynecologic stromal cells | Endometrial adenocarcinoma | 24 | n/d | (Tanwar et al., 2012) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Pten</i> ^{flox/flox} ; <i>Misr2-Cre</i> | Gynecologic stromal cells | Endometrial adenocarcinoma | 9 (100%) | n/d | (Tanwar et al., 2012) |
| <i>Lkb1</i> ^{+flox} ; <i>LSL-Kras</i> ^{G12D} ; <i>Pdx1-Cre</i> | Pancreatic epithelial cells | Pancreatic ductal adenocarcinoma | ~20 | H | (Morton et al., 2010) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Pdx1-Cre</i> | Pancreatic epithelial cells | Pancreatic tumors | ~10 (100%) | n/d | (Morton et al., 2010) |
| <i>Lkb1</i> ^{flox/flox} ; <i>LSL-Kras</i> ^{G12D} ; Adeno-Cre (i.n.) | Pulmonary cells | Metastatic adeno-squamous carcinoma | 9 (56%) | n/d | (Ji et al., 2007) |
| <i>Lkb1</i> ^{flox/flox} ; Lenti-Sox2-Cre (i.n.) | Pulmonary cells | Squamous cell carcinoma | 24-41 (41%) | n/d | (Mukhopadhyay et al., 2014) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Pten</i> ^{flox/flox} ; Adeno-Cre (i.n.) | Pulmonary cells | Squamous cell carcinoma | 40-50 (100%) | n/d | (Xu et al., 2014) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Pten</i> ^{flox/flox} ; <i>Tp53</i> ^{flox/flox} ; Adeno-Cre (i.n.) | Pulmonary cells | Adeno-squamous carcinoma | n/a | n/d | (Xu et al., 2014) |
| <i>Lkb1</i> ^{flox/flox} ; <i>LSL-Kras</i> ^{G12D} ; Adeno-Cre (i.n.) | Pulmonary cells | Adenocarcinoma | n/a | n/d | (Xu et al., 2014) |
| <i>Lkb1</i> ^{flox/flox} ; <i>LSL-Kras</i> ^{G12D} ; <i>Tp53</i> ^{flox/flox} ; Adeno-Cre (i.n.) | Pulmonary cells | Adenocarcinoma | n/a | n/d | (Xu et al., 2014) |

*Latency is shown in weeks, and incidence is indicated in parentheses. LOH, loss of heterozygosity; H, haploinsufficiency; n/d, not determined; n/a, not available; *flox*, flanked by loxP sites; *hypo*, hypomorphic; i.p., intraperitoneal injection; i.u., intrauterine injection; i.v., intravenous injection; i.n., intranasal inhalation; β -NF, β -naphthoflavone; tam, tamoxifen; 4-OHT, 4OH-tamoxifen. The table is established on the basis of the review article (Ollila and Makela, 2011) and expanded with information from the listed references.

Table 1-2. *Lkb1* mouse models with non-tumorigenic phenotypes

| Genotype | Targeting | Phenotypes | References |
|---|-----------------------------|---|--|
| <i>Lkb1</i> ^{-/-} | All tissues | Embryonic death, vascular and neural defects | (Ylikorkala et al., 2001) |
| <i>Lkb1</i> ^{M129G/M129G} (1NMPP1)* | All tissues | Defective organogenesis in lung and pancreas | (Lo et al., 2012) |
| <i>Lkb1</i> ^{flox/-} ; <i>Tie1-Cre</i> | Endothelial cells | Embryonic death, vascular defects | (Londesborough et al., 2008) |
| <i>Lkb1</i> ^{+flox} ; <i>Tie2-Cre</i> | Endothelial cells | Defective revascularization of hind-limb ischemia | (Ohashi et al., 2010) |
| <i>Lkb1</i> ^{flox/flox} ; <i>VE-CAD-Cre</i> | Endothelial cells | Hypertension, cardiac hypertrophy, impaired endothelium-dependent relaxation | (Zhang et al., 2014) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Emx-Cre</i> | Cortical neurons | Defective axon specification | (Barnes et al., 2007) |
| <i>Lkb1</i> ^{+flox} ; <i>AIF</i> ^{flox/flox} ; <i>CamKIIα-Cre</i> | Postmitotic neurons | Increased neuronal cell death following mitochondrial dysfunction | (Germain et al., 2013) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Mx1-Cre</i> ; (i.p., plpC) | Hematopoietic stem cells | Hematopoietic cell death | (Gurumurthy et al., 2010; Nakada et al., 2010) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Rosa26-CreERT2</i> (i.p., tam) | Hematopoietic stem cells | Hematopoietic cell death | (Gan et al., 2010) |
| <i>Lkb1</i> ^{flox/flox} ; <i>MCK-Cre</i> | Cardiac myocytes | Cardiac dysfunction, especially after ischemia | (Jessen et al., 2010; Sakamoto et al., 2006) |
| <i>Lkb1</i> ^{flox/flox} ; <i>α-MHC-Cre</i> | Cardiac myocytes | Cardiac dysfunction, atrial fibrillation, reduced capillaries | (Ikeda et al., 2009) |
| <i>Lkb1</i> ^{flox/flox} ; <i>MCK-Cre</i> | Skeletal myocytes | Impaired contraction-induced glucose uptake | (Sakamoto et al., 2005) |
| <i>Lkb1</i> ^{flox/flox} ; <i>MCK-Cre</i> | Skeletal myocytes | Enhanced glucose tolerance, lower fasting glucose and insulin | (Koh et al., 2006) |
| <i>Lkb1</i> ^{flox/flox} ; <i>MRF4-Cre</i> | Skeletal myocytes | Decreased exercise capacity before training, impaired mitochondrial protein expression after training | (Tanner et al., 2013) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Pdx1-CreER</i> (i.p., tam) | Pancreatic β-cells | Increased β-cell size and insulin secretion, cell polarity defects | (Fu et al., 2009; Granot et al., 2009) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Rip2-Cre</i> | Pancreatic β-cells | Enhanced insulin secretion and glucose tolerance | (Sun et al., 2010) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Rip2-Cre</i> | Thoracic nerves | Axon degeneration, hind-limb paralysis | (Sun et al., 2011) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Adeno-Cre</i> (i.v.) | Hepatocytes | Hyperglycemia, increased gluconeogenesis and lipogenesis | (Shaw et al., 2005) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Alb-Cre</i> | Hepatocytes | Defective bile and cholesterol metabolism | (Woods et al., 2011) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Ah-Cre</i> (i.p., β-NF) | Intestinal epithelial cells | Defective secretory cell differentiation | (Shorning et al., 2009) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Lck-Cre</i> | T cell progenitors | Defective thymocyte survival and differentiation | (Cao et al., 2010; Tamas et al., 2010) |
| <i>Lkb1</i> ^{flox/flox} ; <i>Fabp4 -Cre</i> | Adipose tissue | Defective white adipose tissue growth and differentiation | (Zhang et al., 2013) |

*M129G, mutation in the ATP-binding pocket, which makes the *Lkb1* kinase sensitive to derivatives of the general kinase inhibitor PP1 (e.g., 1NMPP1); *flox*, flanked by loxP sites; i.p., intraperitoneal injection; i.v., intravenous injection; plpC, polyinosinic-polycytidylic acid; tam, tamoxifen; β-NF, β-naphthoflavone. The table is established on the basis of the review article (Ollila and Makela, 2011) and expanded with information from the listed references.

1.3. LKB1 Functions and Its Substrates

LKB1 gene encodes a serine/threonine kinase, which functions as a master upstream kinase, directly phosphorylating and activating AMP-activated protein kinase (AMPK) and 12 AMPK-related kinases (ARKs) (Lizcano et al., 2004) (Figure 1-1 and Table 1-3). By regulating a variety of substrates, LKB1 plays diverse roles in multiple cellular processes, including energy metabolism, proliferation, apoptosis and cell polarity (Alessi et al., 2006; Bright et al., 2009; Vaahhtomeri and Makela, 2011) (Figure 1-1 and Table 1-3). Among those known substrates, AMPK was the first-identified substrate of LKB1 (Hong et al., 2003; Woods et al., 2003), and it is, by far, the best-characterized one (Mihaylova and Shaw, 2011). Many functions of AMPK account for LKB1's role in tumor suppression (Shackelford and Shaw, 2009).

AMPK is an evolutionarily conserved sensor of intracellular energy levels among species, from yeast to humans (Hardie, 2007). It was originally discovered as a mammalian protein kinase that was able to regulate enzymes of lipid metabolism in an AMP-dependent manner (Hardie et al., 1989). In budding yeast, the AMPK orthologue *SNF1* (sucrose non-fermenting 1) gene was initially identified from a screening for mutations that caused defects in metabolism of sucrose (Carlson et al., 1981), and later was functionally related to mammalian AMPK due to sequence homology (Mitchell et al., 1994; Woods et al., 1994). AMPK/Snf1 exists in a heterotrimeric complex with the catalytic α -subunit and regulatory β - and γ -subunits. When intracellular AMP/ADP levels are rising, binding of AMP to the γ -subunit promotes Thr172 phosphorylation at the activation loop of the α -subunit by LKB1 and subsequent allosteric activation of AMPK (Gowans et al., 2013). Under low-energy conditions, activated AMPK restores intracellular ATP levels by stimulating catabolic pathways such as glycolysis and fatty acid oxidation, while inhibiting anabolic pathways such as lipid and protein synthesis (Hardie, 2007). To maintain energy

homeostasis, it rapidly phosphorylates multiple downstream targets, including metabolic enzymes that cause acute metabolic changes, and transcription factors and co-activators that result in adaptive metabolic reprogramming (Figure 1-2) (Hardie, 2007; Mihaylova and Shaw, 2011; Shackelford and Shaw, 2009). Activation of AMPK by LKB1 also suppresses mTOR (mammalian target of rapamycin) complex 1 (mTORC1) by directly phosphorylating TSC2 (tuberous sclerosis 2) and Raptor (regulatory associated protein of mTOR) (Gwinn et al., 2008; Inoki et al., 2003; Shaw et al., 2004a), thereby inhibiting protein translation and cell growth following energy stress. In addition to coordinating cell growth with intracellular energy status via regulation of the mTORC1 pathway, AMPK positively regulates the autophagy cascade by direct phosphorylation of the autophagy initiating kinase ULK1 (Unc-51-like kinase 1) under nutrient restriction (Egan et al., 2011; Kim et al., 2011). Autophagy is an evolutionarily conserved process that degrades and recycles damaged intracellular proteins and organelles in order to provide a source of energy and molecular building blocks (e.g., amino acids) for cell survival in response to various stress, such as limited energy and nutrient supply. In contrast to AMPK, under nutrient-rich conditions, mTORC1 inhibits autophagy by negatively regulating ULK1 and Atg13 (autophagy related 13), two components of the complex that is essential for autophagosome formation (Dunlop and Tee, 2013). Therefore, the coordinated signaling interplay among AMPK, mTORC1 and ULK1 finely orchestrates energy and nutrient homeostasis in cells (Dunlop and Tee, 2013). Therefore, taken together, activation of the LKB1/AMPK pathway provides a survival advantage under energy stress, and inactivation of the LKB1/AMPK pathway renders cells more susceptible to energy stress-induced cell death (Narbonne and Roy, 2009; Shaw et al., 2004b; van der Velden et al., 2011).

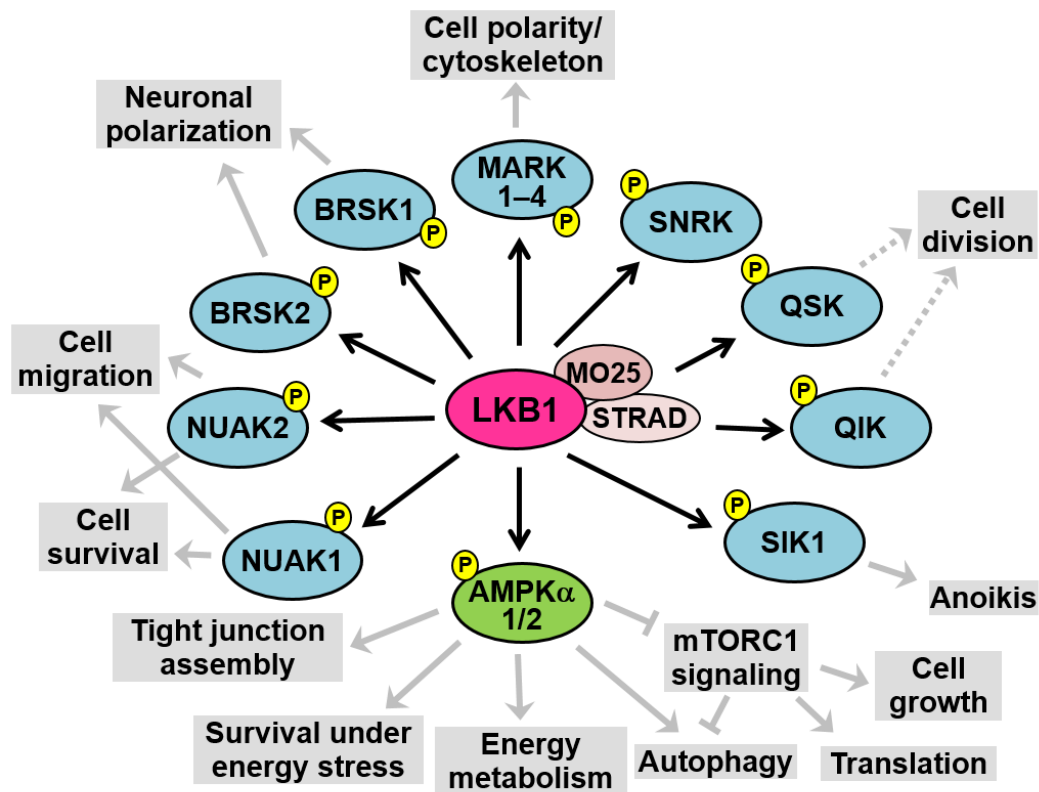


Figure 1-1. A simplified scheme of the LKB1-dependent signaling.

LKB1, existing in a complex with two regulatory proteins, STRAD (Ste20-related adaptor) and MO25 (mouse protein 25), directly phosphorylates and activates a family of AMPK-related kinases that regulate various biological processes (summarized in Table 1-3).

Table 1-3. LKB1 substrates—AMPK-related kinases

| Kinase | Description | Functions | Refs* |
|-------------------|--|---|-------|
| AMPK α 1/2 | AMP-activated protein kinase | Cell metabolism, cell polarity, cell growth, cell survival and autophagy | 1 |
| BRSK1 (SAD-B) | Brain-specific kinase 1 | Neuronal polarization, centrosome duplication | 2 |
| BRSK2 (SAD-A) | Brain-specific kinase 2 | Neuronal polarization | 3 |
| NUAK1 (ARK5) | Novel (nua) kinase 1 | Cancer cell survival and migration, metastasis, terminal axon branching | 4 |
| NUAK2 (SNARK) | Novel (nua) kinase 2 | Cancer cell survival and migration, metastasis | 5 |
| SIK1 | Salt-inducible kinase 1 | p53-dependent anoikis, steroidogenesis, myogenesis, TLR4-mediated signaling, TGF- β signaling | 6 |
| SIK2 (QIK) | Salt-inducible kinase 2 | Adipogenesis, insulin secretion, cardiac hypertrophy, neuronal survival, bipolar mitotic spindle formation | 7 |
| SIK3 (QSK) | Salt-inducible kinase 3 | Chondrocyte hypertrophy, cholesterol and bile acid metabolism, cell division, TLR4-mediated signaling | 8 |
| MARK1–4 | Microtubule affinity-regulating kinases | Cell polarity, neuronal migration, neuronal differentiation, transport, cell-cycle control, Hippo-Yap signaling | 9 |
| SNRK | Sucrose non-fermenting protein (SNF1)-related kinase | Adipocyte inflammation, vascular development, colon cancer cell proliferation, β -catenin signaling | 10 |

*References: 1. (Hardie, 2007), (Mihaylova and Shaw, 2011); 2. (Kishi et al., 2005), (Barnes et al., 2007), (Alvarado-Kristensson et al., 2009); 3. (Kishi et al., 2005), (Barnes et al., 2007); 4. (Sun et al., 2013), (Courchet et al., 2013); 5. (Sun et al., 2013); 6. (Katoh et al., 2004), (Kowanetz et al., 2008), (Cheng et al., 2009), (Stewart et al., 2013), (Yong Kim et al., 2013); 7. (Katoh et al., 2004), (Ahmed et al., 2010), (Sasaki et al., 2011), (Sakamaki et al., 2014), (Popov et al., 2014), (Park et al., 2014); 8. (Sasagawa et al., 2012), (Uebi et al., 2012), (Yong Kim et al., 2013), (Chen et al., 2014); 9. (Matenia and Mandelkow, 2009), (Mohseni et al., 2014); 10. (Chun et al., 2009), (Pramanik et al., 2009), (Rines et al., 2012), (Li et al., 2013).

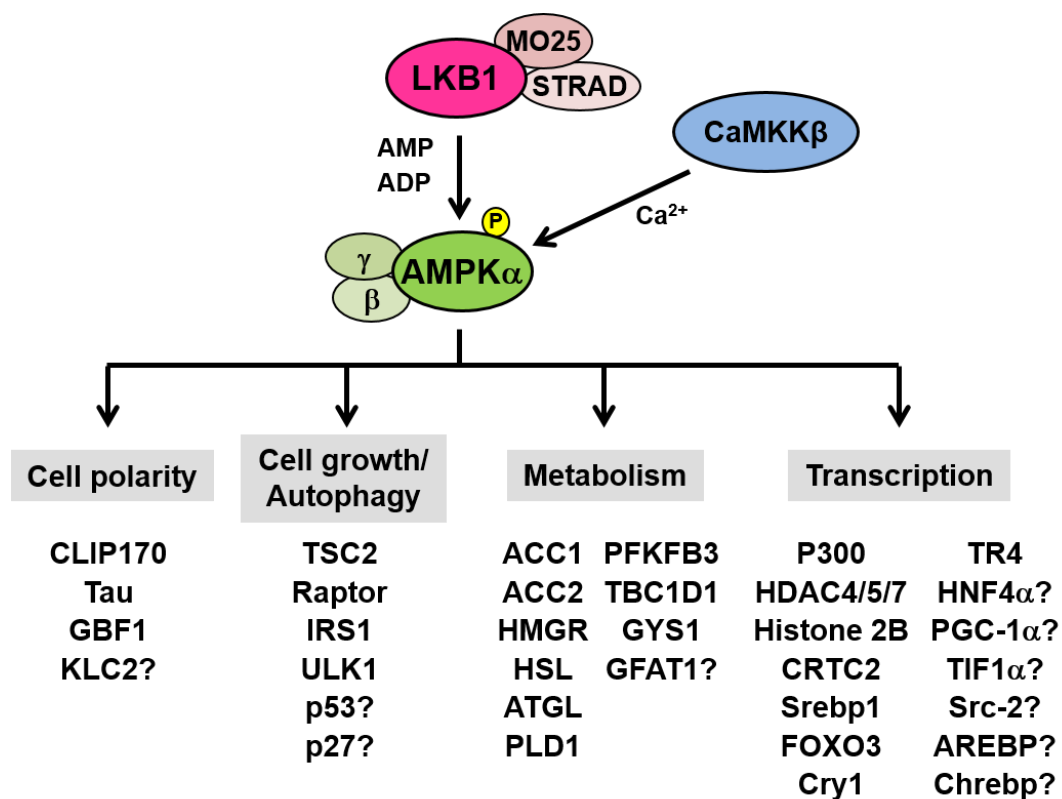


Figure 1-2. The AMPK signaling pathway.

LKB1 and CaMKK β (calmodulin-dependent protein kinase kinase β) are the upstream kinases activating AMPK in response to increase in intracellular levels of AMP (or ADP) and calcium, respectively. Activated AMPK directly phosphorylates a multiplicity of downstream substrates involved in metabolism, cell growth, autophagy, transcription and cell polarity. The well-established substrates are shown, and those that require further *in vivo* validation are shown with a question mark.

1.4. LKB1 Complex

Unlike AMPK and most other kinases that are activated by phosphorylation of their activation loop (close to their catalytic loop), LKB1 is predominantly activated through complex assembly (Boudeau et al., 2004). LKB1 forms a heterotrimeric complex with two accessory proteins, the pseudokinase STRAD (Ste20-related adaptor) and the scaffolding protein MO25 (mouse protein 25) (Baas et al., 2003; Boudeau et al., 2003). The crystal structure study of the heterotrimeric complex by Zeqiraj *et al.* reveals that binding of STRAD and MO25 to LKB1 promotes and stabilizes the activated conformation of LKB1 through a phosphorylation-independent allosteric mechanism (Zeqiraj et al., 2009a), resolving the previous observation of the functional importance of STRAD and MO25 in LKB1 activation (Boudeau et al., 2004; Hawley et al., 2003). Within the LKB1-STRAD-MO25 heterotrimer, there exist considerable interactions among all of the three proteins (Zeqiraj et al., 2009a). STRAD, displaying an active kinase conformation stabilized by association with MO25 (and ATP) (Zeqiraj et al., 2009b), binds LKB1 as a pseudo-substrate (Zeqiraj et al., 2009a). On the other hand, binding of MO25 to LKB1, stabilized by the presence of STRAD, properly positions the LKB1 activation loop in an optimal active conformation competent for phosphorylation of substrates (Zeqiraj et al., 2009a). Accordingly, both STRAD and MO25 are critical for LKB1 kinase activity. Additionally, STRAD, but not MO25, has been demonstrated to facilitate cytoplasmic translocation of LKB1 by serving as an adaptor (Dorfman and Macara, 2008). Nevertheless, to date, how the LKB1-STRAD-MO25 complex is maintained and regulated remains largely unclear.

1.5. Regulation of LKB1

Besides the inherent assembly of the complex as aforementioned, other regulatory mechanisms of LKB1 activation are poorly understood. Thus far, phosphorylation and subcellular localization of LKB1 are thought to be two main regulatory mechanisms of LKB1 signaling. Although several studies have demonstrated that phosphorylation of LKB1 regulates its function in cell-cycle arrest, tumor suppression and cell polarity, such posttranslational modification does not directly impact LKB1 kinase activity (Sebbagh et al., 2011). Several phosphorylation sites on LKB1 have been identified with known or unknown upstream kinases (Figure 1-3). Thr336 of LKB1 is thought to be an autophosphorylation site, and Ser31 of LKB1 can be phosphorylated by an unknown kinase (Sapkota et al., 2002a). However, both phosphorylation sites do not appear to regulate LKB1 activity or localization (Sapkota et al., 2002a). A further study showed that autophosphorylation of LKB1 at Thr336 induces negative feedback control of LKB1 via recruitment of 14-3-3 to LKB1 and subsequent suppression of LKB1 kinase function from binding to its substrates (Bai et al., 2012). It has been reported that LKB1 is phosphorylated by ATM (ataxia telangiectasia mutated) at Thr366 after DNA damage (Sapkota et al., 2002b) and this phosphorylation does not affect LKB1 activity or localization (Sapkota et al., 2002a; Sapkota et al., 2002b). However, this ATM/LKB1 signaling is required for B cell differentiation within germinal centers in response to genotoxic stress (Sherman et al., 2010), and is involved in H₂O₂-induced mTORC1 suppression (Alexander et al., 2010). In B-Raf^{V600E} melanoma cells, phosphorylation of Ser325 and Ser428 of LKB1 by two B-Raf downstream kinases, Erk (extracellular signal-regulated kinase) and p90Rsk (90-kDa ribosomal S6 kinase), respectively, does not affect LKB1 complex integrity but suppresses the ability of LKB1 to bind and activate AMPK, in turn promoting cell proliferation and anchorage-independent growth (Zheng et al., 2009). Nevertheless, it has been

suggested that Ser428 (equivalent to Ser431 in mouse Lkb1) phosphorylation of LKB1 does not appear to directly influence LKB1 kinase activity (Sapkota et al., 2001; Xie et al., 2009), and a recent study demonstrated that both Lkb1 kinase activity and the downstream AMPK activation are not altered in *Lkb1*^{S431A/S431A} knockin mice (Houde et al., 2014). Phosphorylation of LKB1 at Thr307 by PKC- ζ (protein kinase C ζ) regulates nuclear export of LKB1 but not LKB1 kinase activity, consequently directing AMPK activation and suppression of endothelial cell angiogenesis (Xie et al., 2009). Another phosphorylation-mediated spatial regulation of LKB1 has been reported, by which Akt-mediated Ser334 phosphorylation of LKB1 induces the association of LKB1 with 14-3-3 and subsequent nuclear retention of LKB1, in turn inhibiting the tumor-suppressive function of LKB1 (Liu et al., 2012a). LKB1 also undergoes farnesylation at the very C-terminal Cys residue (Cys433 in mouse Lkb1). This modification does not affect LKB1 kinase activity (Houde et al., 2014; Sapkota et al., 2001) but is critical for membrane localization of LKB1 (Houde et al., 2014; Sebbagh et al., 2009). Interestingly, impairment of AMPK activation was observed in *Lkb1*^{C433A/C433A} knockin mice, and a potential mechanism was proposed, whereby Lkb1 farnesylation may promote the co-localization of Lkb1 with the membrane-localized AMPK (as a result of myristoylation of the β -subunit of AMPK (Mitchell et al., 1997; Oakhill et al., 2010)) on the membrane surface and the subsequent AMPK activation (Houde et al., 2014). Another type of LKB1 posttranslational modification—acetylation—has been found. It was shown that acetylation of LKB1 at Lys48 negatively regulates its assembly with STRAD and kinase activity and the deacetylase appears to be SIRT1 (Lan et al., 2008). It is of great interest to address whether other types of posttranslational modification on LKB1 can modulate its activity directly.

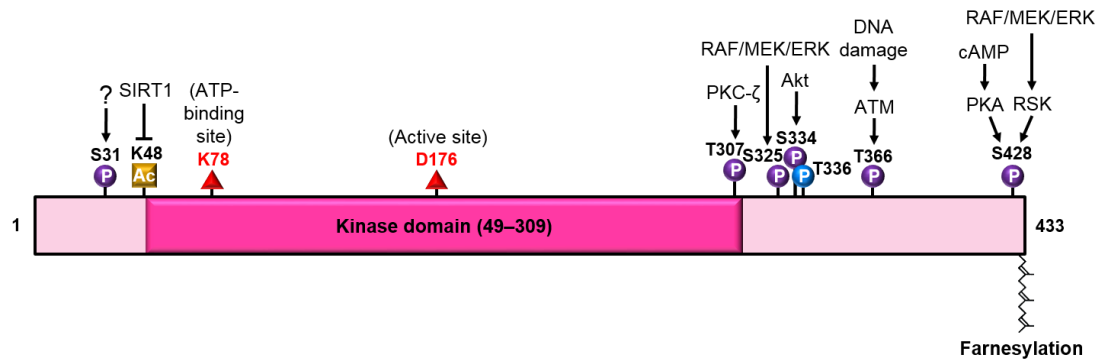


Figure 1-3. The posttranslational modification of LKB1.

The kinase domain of human LKB1 protein is shown and the two residues critical for the kinase activity are indicated by red triangles. The residues where posttranslational modifications take place are indicated with known or unknown upstream regulators. P (in purple or blue circle), phosphorylation; Ac (in square), acetylation. Blue circle, auto-phosphorylation.

1.6. Potential Pro-oncogenic Role of LKB1

Although, as mentioned above, genetic evidence supports the tumor-suppressive role of LKB1, other evidence has revealed that LKB1 may also exhibit previously unrecognized pro-oncogenic functions. Bardeesy *et al.* demonstrated that *Lkb1*-deficient mouse embryonic fibroblasts (MEFs) are resistant to oncogenic transformation (Bardeesy *et al.*, 2002), which may account for the lack of malignant tumors in PJS patients and in many mouse models with *LKB1* deficiency (Bardeesy *et al.*, 2002; Jeon *et al.*, 2012). In UVB-induced murine basal-cell skin carcinoma, upregulated LKB1 expression and AMPK signaling were found concomitant with increased phosphorylation of Akt and GSK3 β (Byekova *et al.*, 2011). In lung and colon cancer cell lines with constitutive Akt activation, LKB1 was shown to be required for Akt-mediated phosphorylation of pro-apoptotic proteins, such as FoxO3a, FoxO1, Bad and GSK3 β , to suppress apoptosis (Zhong *et al.*, 2008). It was demonstrated that LKB1 regulates Akt-mediated cell survival in cells isolated from murine metabolic disorder-derived HCC (Martinez-Lopez *et al.*, 2010). Also, elevated LKB1 expression was observed in murine and human HCC (Martinez-Lopez *et al.*, 2012; Martinez-Lopez *et al.*, 2010), and LKB1 silencing attenuates the *in vivo* growth of mouse hepatoma cells (Martinez-Lopez *et al.*, 2012). Moreover, Jeon *et al.* demonstrated that knockdown of LKB1 or AMPK in breast cancer cells attenuates xenograft tumor growth due to failure in inhibition of acetyl Co-A carboxylase (ACC) and maintenance of intracellular NADPH levels (Jeon *et al.*, 2012). The discrepancy among studies showing a tumor-suppressive or a pro-oncogenic role for LKB1 suggests that LKB1 appears to be a “double-edged sword” in terms of tumor suppression and promotion, and which role LKB1 plays may be determined by distinct cell contexts, tissue types, and nutrient availability within tumor microenvironments. Because tumors mostly reside in a metabolic stress environment, it is possible that

cancer cells could use LKB1/AMPK signaling for their survival. Whether LKB1 has a stage-specific function in cancer progression remains to be determined.

1.7. Ubiquitination and Its Function

Ubiquitination is a type of posttranslational modification by which a highly conserved small regulatory protein ubiquitin (a 76-amino acid polypeptide of ~8.5 kDa) is covalently attached to one or multiple lysine residues of a target protein, termed monoubiquitination or multi-monoubiquitination, respectively. Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), and any of them can be utilized for ubiquitin chain formation, for example, K63-linked polyubiquitin chains (Figure 1-4). In other words, ubiquitination has varied layouts or forms (Adhikari and Chen, 2009; Komander, 2009). It is therefore conceivable that, compared with other types of posttranslational modification, ubiquitination plays a more complicated role in determining the fate of target proteins and thereby functions in a great diversity of cellular processes. So far, K48-linked and K63-linked ubiquitin chains are best characterized. Classically, K48-linked polyubiquitin chains are the major targeting signal for 26S proteasome- dependent proteolysis, whereas K63-linked ubiquitination has non-proteolytic functions in numerous cellular events, such as DNA damage repair and intracellular signaling (Adhikari and Chen, 2009; Komander, 2009). The ubiquitination reaction involves three classes of enzymes for a three-step enzymatic cascade, namely ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (UBCs) (E2s) and ubiquitin ligases (E3s) (Figure 1-5). Basically, ubiquitin is activated by the E1 and transferred to the E2, and then the E3 bridges the substrate to the 'charged' E2 for ubiquitin transfer. E2 enzymes are also capable of catalyzing ubiquitin chain formation and determining the linkage types of ubiquitin chains (Ye and Rape, 2009). E3 ligases are responsible for substrate specificity, and can be

categorized into three major classes based on their structural domains and mechanistic function in ubiquitin transfer: RING (really interesting new gene) E3s, HECT (homologous to E6-associated protein C-terminus) E3s and RBR (RING-between-RING) E3s (Berndsen and Wolberger, 2014). On the other hand, ubiquitin addition can be removed from the target proteins by a family of deubiquitinating enzymes (DUBs). Hence, the ubiquitination/deubiquitination cycle can orchestrate a number of non-proteolytic processes in cells, like transcription, signaling transduction and vesicle trafficking. There are two E1s, at least 38 E2s, 600-1000 E3s, and approximately 79 putative DUBs encoded in the human genome (Nijman et al., 2005; Ye and Rape, 2009).

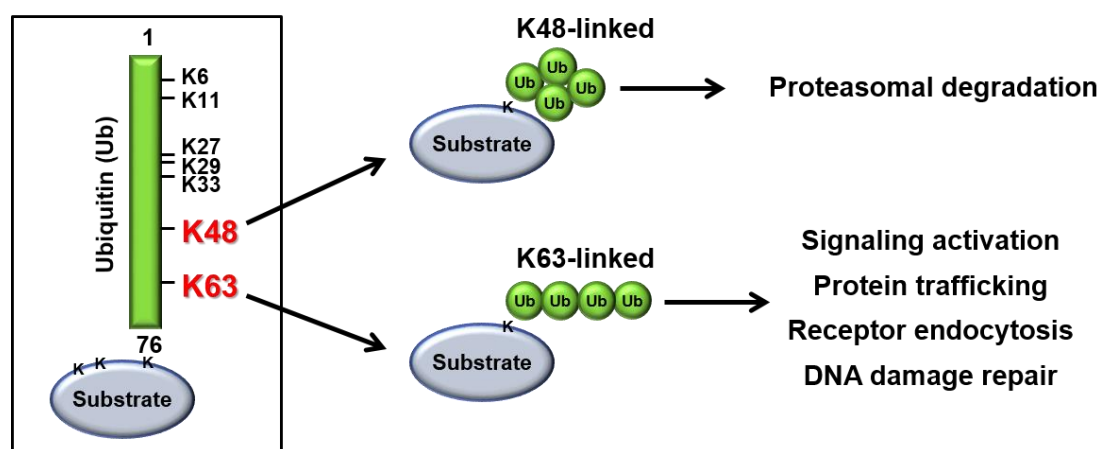


Figure 1-4. A simplified scheme of ubiquitin and its lysine residues.

A ubiquitin molecule is attached to a lysine (K) residue of a substrate through its last residue (Gly76). The seven lysine residues of ubiquitin, all of which can be subjected to polyubiquitination, are indicated and the most characterized two are in red. The schematic structures of K48-linked and K63-linked polyubiquitin chains are shown and their biological functions are indicated.

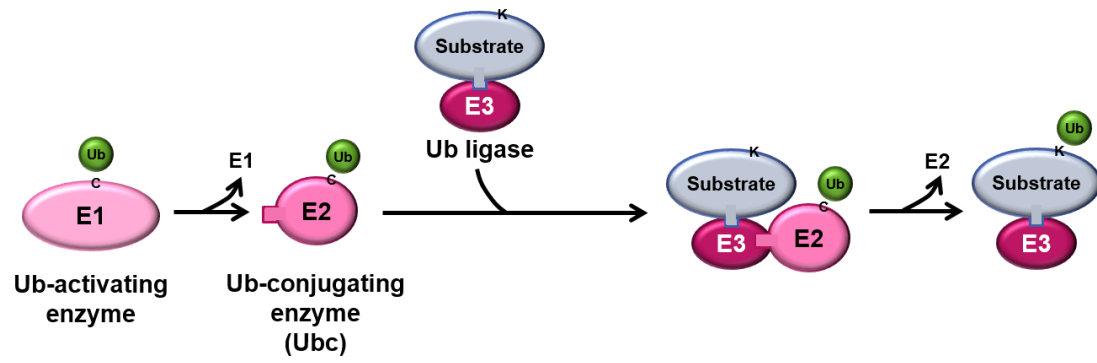


Figure 1-5. A simplified scheme of a ubiquitination reaction.

Basically, a ubiquitin molecule is activated by a ubiquitin-activating enzyme (E1) and transferred to a ubiquitin-conjugating enzyme (E2). Then a ubiquitin ligase (E3) bridges the substrate to the 'charged' E2 for ubiquitin transfer.

1.8. Skp2 and Skp2-SCF Ubiquitin Ligase Complex

A subset of multi-subunit RING E3 ligases are known as the cullin-RING ligases (CRLs). Basically, they consist of a scaffold protein cullin (CUL), a RING-containing enzyme Rbx1/2 (RING-box 1 or 2; also known as Roc1/2 (regulator of cullin 1 or 2)) and an adaptor protein, called F-box protein (Berndsen and Wolberger, 2014). There are 7 cullins (Cul1, 2, 3, 4a, 4b, 5, and 7) and 69 F-box proteins identified in humans (Lee and Diehl, 2014). The best characterized CRLs are the Skp1-Cul1-F-box (SCF) ubiquitin ligases (Lee and Diehl, 2014). Within SCFs, Cul1 provide the scaffold for docking the adaptor Skp1 (S-phase kinase-associated protein 1) and Rbx1, which drags E2s carrying activated ubiquitin. Skp1 further links an F-box protein, which functions as the substrate recognition component, to the ligase complex. Through their F-box domain (approximately 40 amino acids) in the N-terminal region, F-box proteins bind to Skp1, and on the basis of their substrate-interacting domains in the C-terminal region, F-box proteins are classified into three subtypes: FBXWs (F-box proteins with WD40 domains), FBXLs (F-box proteins with leucine-rich repeats (LRR)) and FBXOs (F-box proteins with other diverse domains) (Frescas and Pagano, 2008). So far, only a small number of F-box proteins have been well characterized with known substrates, including β -TrCP (β -transducin repeat-containing protein 1 and 2; also known as FBXW1 and FBXW11, respectively), Fbxw7 and Skp2 (also known as FBXL1) (Figure 1-6A) (Chan et al., 2010b). Likewise, another multi-subunit ubiquitin ligase complex—the anaphase promoting complex/cyclosome (APC/C)—requires its activator proteins, Cdc20 (cell division cycle 20 homolog) and Cdh1 (cadherin 1) for substrate recognition and full function (Frescas and Pagano, 2008).

The Skp2-SCF ubiquitin ligase complex is composed of Skp2, Skp1, Cul1 and Rbx1 (Figure 1-6B), and capable of targeting its substrates for ubiquitination and subsequent proteasome-dependent degradation. In this regard, many Skp2

substrates have been demonstrated (Table 1-4); however, the physiological relevance of most of them in Skp2-mediated functions remains to be determined. By contrast, the cyclin-dependent kinase inhibitor p27 (also known as Kip1) is identified to be the major physiological and pathological substrate of Skp2 (Chan et al., 2010b). The defects of *Skp2* deficiency, including reduced cell proliferation in MEFs, and reduced tissue mass, organ size and body weight in mice, can be rescued by double deficiency of *p27* and *Skp2* (Cooke et al., 2007; Nakayama et al., 2004). Moreover, overexpression of Skp2 has been observed in association with the inverse expression of p27 in multiple human cancers for poor prognosis of patients (Chan et al., 2010b; Frescas and Pagano, 2008).

Besides its well-characterized proteolytic function, the Skp2-SCF has been demonstrated to exhibit non-proteolytic function in regulating its substrates (Table 1-4). Our group showed that Skp2-SCF-mediated K63-linked ubiquitination of Nbs1 (Nijmegen breakage syndrome 1; also known as nibrin) is critical for Nbs1-mediated ATM activation during DNA double-strand breaks, which may account for Skp2's role in homologous recombination repair (Wu et al., 2012). Our group also reported that the Skp2-SCF is a critical ubiquitin ligase specific for EGF-induced ubiquitination and activation of Akt and regulates glycolysis, Herceptin sensitivity and breast cancer progression (Chan et al., 2012), in contrast to the role of Traf6 (tumor necrosis factor (TNF) receptor-associated Factor 6) ubiquitin ligase in IGF-induced Akt ubiquitination and activation (Yang et al., 2009). In addition, Skp2 functions independently of SCF (Figure 1-6C). Kitagawa *et al.* showed that Skp2 suppresses apoptosis by sequestering p300 from p53, by which acetylation of p53 and subsequent activation of p53 transcriptional activity are repressed (Kitagawa et al., 2008). Also, our group demonstrated that Skp2, acting as a co-activator along with Myc, p300 and Miz1 (Myc-interacting zinc finger protein 1) for RhoA (Ras homolog gene family, member A)

transcription, regulates cell migration, invasion and cancer metastasis (Chan et al., 2010a). In mice, *Skp2* deficiency results in several metabolically-related defects, such as reduced subcutaneous and visceral fat pad mass and adipocyte number (Cooke et al., 2007). Additionally, loss of *Skp2* causes hypoinsulinemia, glucose intolerance, and resistance to high fat diet-induced or *lethal yellow agouti* (*A^y*) mutation-induced obesity (Sakai et al., 2007; Zhong et al., 2007). These reports suggest a role of *Skp2* in establishing adipose and pancreatic β -cell mass. Although those authors attributed the phenotypes to accumulation of p27, one study inconsistently showed that *Skp2* controls adipogenesis via a p27-independent mechanism in primary MEFs (Okada et al., 2009). Accordingly, further research is required for elucidating the underlying mechanisms by which *Skp2* is associated with those metabolic alterations. Taken together, these findings implicate that future investigations may be needed to identify 'other' unrecognized substrates or functions of *Skp2* dependent or independent of SCF.

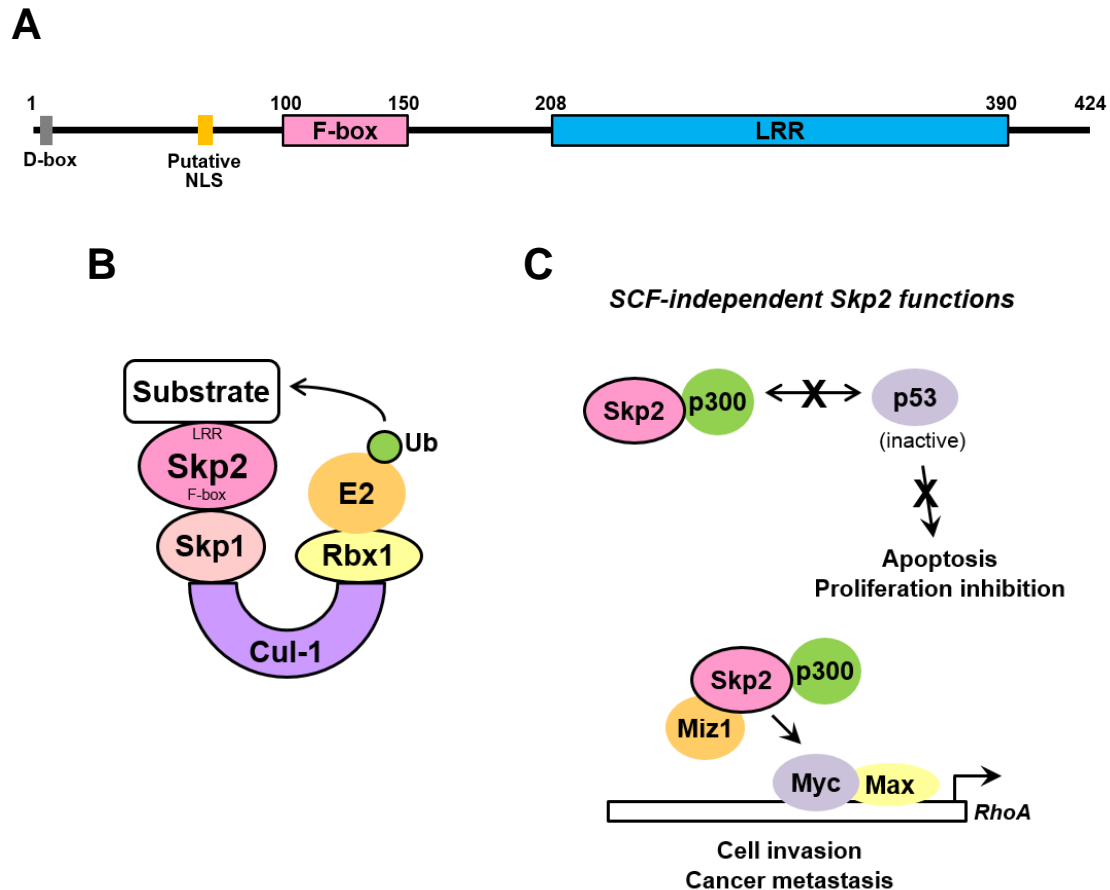


Figure 1-6. Schematic diagrams of Skp2 domain structure and functions.

(A) Functional domains of human Skp2 are shown. Destruction box (D-box) (amino acids 3–6) is required for Skp2 degradation mediated by Cdh1-APC ubiquitin ligase. A putative nuclear localization sequence (NLS) is located from amino acids 66–72.

(B) The Skp2-SCF ubiquitin ligase complex is shown. Ubiquitin (Ub) is transferred from the E2 to the substrate.

(C) A simple scheme of SCF-independent functions of Skp2.

Table 1-4. The known Skp2-SCF substrates

| Reported substrate(s) | Substrate function(s) |
|--|--|
| Through proteolytic regulation* | |
| p27, p21, p57, E2F-1, MEF, p130, Tob1, Cyclin D1, Cyclin E, Smad4, Myc, Myb, RASSF1A | Cell cycle control |
| E2F-1, Myc, FoxO1 | Apoptosis |
| Orc1, Cdt1 | DNA replication |
| Rag2 | DNA recombination |
| Brca2 | DNA repair |
| Cdk9 | Transcriptional elongation |
| MKP1 | Erk signaling |
| UBP43 | Type 1 interferon signaling |
| Through non-proteolytic regulation | |
| Nbs1† | ATM activation, DNA repair |
| Akt‡ | Glycolysis, Herceptin sensitivity, tumorigenesis |

*The information about the Skp2-SCF substrates undergoing proteolysis is adopted from the reference (Chan, 2010). †The reference (Wu, 2012). ‡ The reference (Chan, 2012).

1.9. Rationale and Hypothesis

Many essential, basic cellular processes (e.g., cell division, DNA repair, and gene expression control) are conserved across different species, even between yeast and humans. The SCF ubiquitin ligase complex is evolutionally conserved from yeast to humans. In yeast, it is composed of Skp1, Cdc53 (cullin) and one of the three F-box proteins, Cdc4 (cell division control protein 4), Grr1 (glucose repression-resistant 1) or Met30 (methionine-requiring protein 30) (Patton et al., 1998). Yeast Grr1, which contains LRR (Flick and Johnston, 1991), appears to be organized similarly to human Skp2. It regulates cell cycle progression and cellular metabolism through ubiquitin-mediated protein degradation (Benanti et al., 2007; Li and Johnston, 1997), and plays a role in morphogenesis as Elm1p (Blacketer et al., 1995), the Snf1 (yeast AMPK counterpart) kinase closely related to LKB1 (Sutherland et al., 2003). It is found that in mammals, *Skp2*-deficient MEFs and *LKB1*-deficient MEFs both exhibit resistance to oncogenic transformation (Bardeesy et al., 2002; Lin et al., 2010), and both proteins were shown to regulate maintenance of hematopoietic stem cells (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010; Wang et al., 2011). Moreover, *Skp2* deficiency caused hyperglycemia in mice (Zhong et al., 2007), which was also observed in mice with *LKB1* deletion in the liver (Shaw et al., 2005). All these imply that Skp2 may be linked to the LKB1/AMPK pathway. Therefore, in this dissertation study, we aimed to explore the potential relationship between Skp2 and the LKB1-AMPK pathway and to decipher the mechanism by which LKB1 kinase activity is maintained.

Chapter 2

Materials and Methods

2.1. Cell Culture and Reagents

Wild-type (WT) and *Skp2*-knockout (*Skp2*-KO) mouse embryonic fibroblasts (MEFs) were prepared from mice as previously described (Lin et al., 2010). All manipulations were performed under Institutional Animal Care and Use Committee approval protocol. *LKB1*-knockout (*LKB1*-KO) MEFs were a kind gift from Dr. Nabeel Bardeesy (Harvard Medical School) (Bardeesy et al., 2002). BT-474, MCF-7, Hep3B, HEK293, HEK293T, HeLa and A549 cells were obtained from American Type Culture Collection. BT-474 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), and all the other cells were cultured in DMEM supplemented with 10% FBS. For glucose deprivation, cells were washed with PBS once and then incubated in glucose-free DMEM (Invitrogen) supplemented with 10% dialyzed FBS (Sigma) for the indicated times. 2-deoxyglucose (2-DG), 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and phenformin were purchased from Sigma.

2.2. Plasmids

His₆-ubiquitin (His-Ub), His₆-ubiquitin-K48R (His-Ub-K48R), His₆-ubiquitin-K63R (His-Ub-K63R), pcDNA4-Xpress-Skp2 (Xp-Skp2), pcDNA4-Xpress-Skp2-NES (Xp-Skp2-NES), pcDNA3-Flag-Skp2, pBabe-Skp2 and pBabe-H-RasG12V constructs were described previously (Chan et al., 2012; Lin et al., 2010; Lin et al., 2009). pcDNA3-Flag-LKB1, pcDNA3-Flag-LKB1-KD (kinase-dead; K78I), pBabe-Flag-LKB1, pBabe-Flag-LKB1-KD and pcDNA4-Xpress-STRAD (Xp-STRAD) constructs were purchased from Addgene. pET30a-AMPK α_{1-312} (His-AMPK α_{1-312}) construct was a kind gift from Dr. Gary Lopaschuk (University of Alberta, Canada) (Altarejos et al., 2005). pSG-Flag-LKB1 constructs expressing various LKB1 fragments were kindly provided by Dr. Christelle Forcet (Institut de Génomique Fonctionnelle de Lyon, France) (Nony et al., 2003). pcDNA3-HA-AMPK α_1 and pcDNA3-HA-AMPK α_2 constructs were a kind

gift from Dr. Kun-Liang Guan (University of California, San Diego) (Inoki et al., 2003). The LKB1 constructs including pCMV-myc-LKB1, pcDNA4-Xpress-LKB1 (Xp-LKB1 with various LKB1 fragments), pWZL-myc-LKB1 and pGEX-5X-1-LKB1 (GST-LKB1) were cloned from pcDNA3-Flag-LKB1 (Tables 2-1 and 2-2). All LKB1-K-to-R mutant constructs were generated from the WT LKB1 constructs by using the PCR-based site-directed mutagenesis method. To re-express LKB1 that cannot be targeted by LKB1 shRNA in cells with stable LKB1 knockdown, silent mutations were generated on LKB1 constructs by using the PCR-based site-directed mutagenesis method (Table 2-2; sm, silent mutation).

Table 2-1. Cloning information of the LKB1 constructs

| Construct Name | Primer pairs | Cloning Sites |
|--------------------------|--------------------------------|---------------------------|
| pCMV-myc-LKB1 | LKB1+1-HindIII-F & LKB1-XhoI-R | HindIII/HindIII-XhoI/XhoI |
| pGEX-5X-1-LKB1 | EcoRI-LKB1-F & XhoI-LKB1-R | EcoRI/EcoRI-XhoI/XhoI |
| pWZL-myc-LKB1 | Myc-LKB1-F & LKB1-EcoRI-R | EcoRI/EcoRI-EcoRI/EcoRI |
| pcDNA4-Xp-LKB1 aa1-87 | LKB1-FL-F & LKB1-87-R | T/A cloning |
| pcDNA4-Xp-LKB1 aa243-433 | LKB1-243-F & LKB1-FL-R | T/A cloning |
| pcDNA4-Xp-LKB1 aa317-433 | LKB1-317-F & LKB1-FL-R | T/A cloning |

Table 2-2. Primer sequences

| Primer Name | Primer sequence (5' to 3') |
|------------------|--|
| LKB1+1-HindIII-F | ATTAAAGCTTATGGAGGTGGTGGACCCGCAG |
| LKB1-XhoI-R | ATTACTCGAGTCACTGCTGCTTGCAGGCCGACAG |
| EcoRI-LKB1-F | CCGAATTCATGGAGGTGGTGGACCCGCAGCAGC |
| XhoI-LKB1-R | CTCTCGAGTCACTGCTGCTTGCAGGCCGACAG |
| Myc-LKB1-F | CGCGAATTCATGGAACAAAACTTATTTCTGAAGAAGATCTGGAG GTGGTGGACCCGCAGCAGCTGGGCATGTTC |
| LKB1-EcoRI-R | ATATGAATTCTCACTGCTGCTTGCAGGCCGACAG |
| LKB1-FL-F | ATGGAGGTGGTGGACCCG |
| LKB1-FL-R | TCACTGCTGCTTGCAGGCC |
| LKB1-87-R | CTACCTTCGCAACTTCTTCTTCTTG |
| LKB1-243-F | GTCACCCTCTACAACATCACC |
| LKB1-317-F | GAAGCACCAGTGCCCATCCC |
| smLKB1-5-F | GGATGACATCATCTACACACAAGATTTACGGTGCCCGGACAGG |
| smLKB1-5-R | CCTGTCCGGGCACCGTGAAATCTTGTGTGTAGATGATGTCATCC |

2.3. Transfection and Viral Infection

For transfection of HEK293 or HEK293T cells with exogenous plasmids, the calcium phosphate transfection method was used. For control, Skp2, LKB1, Ubc13, or Ubc5C knockdown, the indicated cell lines were infected with lentiviruses packaged from HEK293T cells transfected with pLKO.1-puro-shRNA constructs (Sigma) and packaging plasmids (Chan et al., 2012). The lentiviral shRNAs used are listed in Table 2-3. For transduction of vector, LKB1, LKB1-KD, LKB1-5KR, Skp2 or H-RasG12V, the indicated cell lines were infected with retroviruses packaged from HEK293T cells transfected with pBabe-puro constructs and packaging plasmids (Chen et al., 2011). The stably infected cells were selected by 1, 1.5 or 2 µg/ml puromycin for 4-7 days. For transduction of LKB1-shRNA non-targeting myc-LKB1, control- or LKB1-knockdown Hep3B cell lines were infected with retroviruses packaged from HEK293T cells transfected with pWZL-myc-smLKB1 constructs and packaging plasmids (Chen et al., 2011). The stably infected cells were selected by 100 µg/ml hygromycin for 7 days.

Table 2-3. Lentiviral shRNAs used in the study

| shRNA | Sequence (5' to 3') |
|--------------------------|----------------------------|
| Luciferase shRNA control | CGCTGAGTACTTCGAAATGTC |
| Skp2 shRNA-1 | GATAGTGTGTCATGCTAAAGAAT |
| Skp2 shRNA-2 | GCCTAAGCTAAATCGAGAGAA |
| LKB1 shRNA | CATCTACACTCAGGACTTCAC |
| UbcH5c shRNA | CCTGCATTATAGCTGGAATAA |
| Ubc13 shRNA | CCTTCCAGAAGAATACCCAAT |

2.4. Immunoblotting, Immunoprecipitation and Antibodies

For immunoblotting analysis, cells were lysed by direct resuspension in RIPA buffer (50mM Tris-HCl [pH 8.0], 150mM NaCl, 5mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% (v/v) NP-40, 50mM NaF, 10mM sodium pyrophosphate, 10mM

disodium glycerophosphate, protease inhibitor cocktail), and then clarified cell lysates were subjected to SDS-PAGE followed by immunoblotting. For protein quantification, the Bradford protein assay was applied using Bio-Rad protein assay dye reagent (Bio-Rad). For immunoprecipitation, cells were lysed in RIPA directly, or E1A buffer (50mM HEPES [pH 7.5], 250mM NaCl, 5mM EDTA, 0.1% (v/v) NP-40, protease inhibitor cocktail) followed by sonication. Clarified cell lysates were incubated with the indicated antibodies overnight, and then protein A/G beads (Santa Cruz Biotechnology) were added for 3-5 hours. Beads were washed four times with RIPA or E1A buffer. Proteins were eluted in 2X SDS-sample buffer and subjected to immunoblotting analysis. Antibodies used are listed in Table 2-4.

Table 2-4. Antibodies used in the study

| Antibody | Company | IB (v/v)[†] | IP (w/w)[‡] |
|-------------------------------|------------------------------|-----------------------------|-----------------------------|
| anti-ACC | Cell Signaling | 1:1000 | |
| anti-p-ACC (Ser79) | Cell Signaling | 1:5000 | |
| anti-AMPK α | Cell Signaling | 1:2000 | |
| anti-p-AMPK α (Thr172) | Cell Signaling | 1:1000 | |
| anti-Cul1 | Invitrogen | 1:1000 | |
| anti-Erk1/2 | Cell Signaling | 1:2000 | |
| anti-p-Erk1/2 (Thr202/Tyr204) | Cell Signaling | 1:2000 | |
| anti-Flag | Sigma | 1:5000 | 1:1000 |
| anti-Grp78 | BD Transduction Laboratories | 1:5000 | |
| anti-HA | Covance | 1:10000 | |
| anti-Lamin B1 | Abcam | 1:5000 | |
| anti-LKB1 | Santa Cruz Biotechnology | 1:2000 | 1:500 |
| anti-MO25 | Cell Signaling | 1:5000 | |
| anti-Myc | Santa Cruz Biotechnology | 1:1000 | |
| anti-Raptor | Cell Signaling | 1:1000 | |
| anti-p-Raptor (Ser792) | Cell Signaling | 1:1000 | |
| anti-pan-Ras | Calbiochem | 1:2000 | |
| anti-Skp1 | BD Transduction Laboratories | 1:1000 | |
| anti-Skp2 | Invitrogen | 1:2000 | 1:1000 |
| anti-Ubc5C | Cell Signaling | 1:2000 | |
| anti-Ubc13 | Invitrogen | 1:2000 | |
| anti-ubiquitin | Santa Cruz Biotechnology | 1:1000 | |
| anti-Xpress | Invitrogen | 1:5000 | |
| anti- α -tubulin | Sigma | 1:10000 | |
| anti- β -actin | Sigma | 1:20000 | |

[†]IB, immunoblotting; antibody dilution at the volume-to-volume (v/v) ratio.

[‡]IP, immunoprecipitation; antibody dilution at the weight-to-weight (w/w) ratio.

2.5. Recombinant Protein Purification

Recombinant His-AMPK α_{1-312} or GST-LKB1 (WT or 5KR) protein was expressed in transformed BL21 bacteria by induction at 25°C for 18 hours with 250 μ M isopropyl- β -D-thiogalactopyranoside. The bacteria were lysed by freeze-thawing and sonication, and the bacterial lysates were subjected to protein purification. His-AMPK α_{1-312} was purified by using a nickel-agarose column (Invitrogen) followed by elution with a step gradient of imidazole, and GST-LKB1 (WT or 5KR) was purified by using glutathione-S-agarose beads (Invitrogen) followed by elution with reduced glutathione (Fisher Scientific). The eluted proteins were concentrated and desalted by centrifugation using 10-kDa-cutoff Centricon (Millipore).

2.6. In Vitro LKB1 Kinase Assay

Purified recombinant His-AMPK α_{1-312} protein was used as the LKB1 substrate. Endogenous LKB1 or exogenous Flag-LKB1 immunoprecipitated from cells by antibodies against LKB1 or Flag and protein A/G beads, or recombinant GST-LKB1 (WT or 5KR) purified from bacteria was incubated with recombinant His-AMPK α_{1-312} at 30°C for 20 or 30 minutes in 20 μ l of reaction buffer (25mM Tris-HCl [pH 7.5], 5mM β -glycerophosphate, 2mM DTT, 0.1mM Na₃VO₄, 10mM MgCl₂, 0.5mM ATP). After incubation, proteins were eluted in SDS-sample buffer and subjected to immunoblotting analysis. LKB1 kinase activity was directly determined by measuring Thr172 phosphorylation of the recombinant AMPK α_{1-312} by using anti-phospho-AMPK α (Thr172) antibody.

2.7. In Vivo and In Vitro Ubiquitination Assays

In vivo and *in vitro* ubiquitination assays were performed as described previously (Chan et al., 2012; Yang et al., 2009). For the *in vivo* ubiquitination assay, HEK293T

or HEK293 cells were transfected with His-Ub and the indicated plasmids for 48 hours and lysed by using high-stringency denaturing buffer (6M guanidine-HCl, 0.1M Na₂HPO₄/NaH₂PO₄, and 10mM imidazole at pH 8.0). The cell extracts were then incubated with nickel beads (Invitrogen) for 3 hours, washed, and subjected to immunoblotting analysis. For the *in vitro* ubiquitination assay, the SCF/Flag-Skp2 complex was expressed in HEK293T cells, immunoprecipitated by using anti-Flag antibody, and eluted from protein A/G beads by using Flag peptides according to the manufacturer's standard procedures (Sigma). The purified SCF/Flag-Skp2 and recombinant GST-LKB1 were incubated at 37°C for 3 hours in 20 µl of reaction buffer (20mM HEPES [pH 7.4], 10mM MgCl₂, 1mM DTT, 59mM ubiquitin, 50nM E1, 850nM Ubc13/Uev1a, and 1mM ATP). After incubation, the protein mixtures were diluted in RIPA buffer and subjected to immunoprecipitation using anti-Flag antibody overnight and protein A/G beads for an additional 3 hours. Then beads were washed four times with RIPA buffer, and proteins were eluted in SDS-sample buffer and subjected to immunoblotting analysis.

2.8. Preparation of Cytosolic and Nuclear Fractions

Cells were harvested and resuspended in hypotonic buffer (10mM Tris-HCl [pH 7.6], 10mM MgCl₂, 0.1% (v/v) NP-40, protease inhibitor cocktail) followed by Dounce homogenization. The suspensions were centrifuged at 1,300 x g for 5 minutes at 4°C, and then the resulting supernatants and nuclear pellets were further processed for cytosolic and nuclear fractions, respectively. For cytosolic fractions, the supernatants were clarified by centrifugation at 13,500 rpm for 15 minutes at 4°C. For further immunoprecipitation, the concentration of NP-40 in the cytosolic fractions was adjusted from 0.1% to 1%. For nuclear fractions, the nuclear pellets were washed once with hypotonic buffer and then resuspended in RIPA buffer followed by

sonication and centrifugation.

2.9. Cell Viability Assay

For *Skp2*-KO and *LKB1*-KO MEFs, viable and dead cells were counted directly under the microscope using the trypan blue exclusion assay. For glucose-starved A549 and Ras-overexpressing Hep3B cells, cell death was measured using DAPI staining as previously described (Jeon et al., 2012). Briefly, cells were seeded at a low density in 12-well plates in triplicates overnight and then subjected to glucose deprivation. The treated cells were fixed by directly adding formaldehyde (final concentration 12%) to the culture medium. After overnight fixation at 4°C, the cells were stained with DAPI (1 µg/ml) for 5 minutes, and then washed and left in PBS. Under the fluorescence microscope, compared with the untreated cells, the treated cells with condensed or fragmented nuclei were counted as dead cells. For administration of phenformin or/and *Skp2* inhibitor (compound #25), $1.2\text{--}1.5 \times 10^4$ of cell were seeded in 12-well plates in triplicates for 24 hours and then treated with the indicated drug(s) at the indicated concentrations for 3 days. Drugs were refreshed once after treatment for 2 days. Viable cells were counted under the microscope using the trypan blue exclusion assay.

2.10. Detection of Damaged Mitochondria

Mitochondria were analyzed by MitoTracker staining followed by flow cytometry analysis as described previously (Tal et al., 2009). Cells were co-stained with MitoTracker Green (for total mitochondria) and MitoTracker Red (for live or respiring mitochondria) at 100 nM in the culture medium for 20-30 minutes at 37°C. After staining, the cells were washed, harvested and then resuspended in PBS for flow cytometry analysis. The FITC-positive and PE-negative/low populations were gated

as defective mitochondria.

2.11. In Vivo Tumorigenesis Assay

3x10⁶ of stable Hep3B cells with LKB1 overexpression were subcutaneously injected into the flanks of nude mice. The tumor size was measured by the caliper, and the tumor volume (in mm³) was calculated by the equation: volume = (width)² x length/2. All animal experiments were performed under Institutional Animal Care and Use Committee approval protocol. For further immunohistochemistry, isolated xenograft tissues were fixed in 10% formalin and embedded in paraffin in accordance with standard procedures.

2.12. Patients, Tissue Specimens and Immunohistochemistry

This retrospective study had been approved by the Institutional Review Board of the Chi-Mei Medical Center. Immunoexpression was assessed on 120 consecutively treated primary hepatocellular carcinoma underwent surgical resection with curative intent between 1997 and 2002. The clinicopathologic variables evaluated from the 120 patients are listed in Table 3-1. The procedures of immunohistochemistry were identical to previously described (Chan et al., 2012). The slides were incubated with primary antibodies against Skp2 (1:100; Zymed) and LKB1/STK11 (1:50; Epitomics), respectively. Primary antibodies were detected using the ChemMate DAKO EnVision kit (DAKO, K5001). The slides were incubated with the secondary antibody for 30 minutes and developed with 3,3-diaminobenzidine for 5 minutes. Immunostaining was scored by two pathologists (Drs. Chien-Feng Li and Hsuan-Ying Huang) by using a multiheaded microscope to reach a consensus for each case on the H-score as previously described (Chan et al., 2012).

2.13. Statistical Analysis

All data are shown as means \pm s.d. for at least three independent experiments, unless otherwise indicated. Statistical significance was determined by unpaired two tailed Student's t-tests, and *P*-values less than 0.05 were considered statistically significant.

Chapter 3

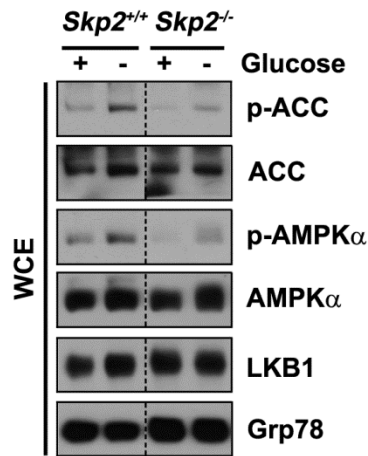
Results

3.1. *Skp2* Regulates the LKB1/AMPK Pathway

To determine whether Skp2 is involved in the LKB1/AMPK pathway, we first examined activation of the LKB1/AMPK signaling induced by energy stress in Skp2-deficient cells. Phosphorylation of ACC at Ser79, a well-established readout of AMPK activity, was analyzed in *Skp2*-KO primary MEFs and various cell lines with Skp2 knockdown under glucose deprivation condition or treatment with glycolysis inhibitor 2-deoxyglucose (2-DG) where intracellular AMP levels were increased. As expected, phosphorylation of ACC as well as AMPK α was induced by glucose deprivation in *wild-type* (WT) primary MEFs; however, in *Skp2*-KO MEFs, glucose deprivation induced much lower levels of ACC and AMPK α phosphorylation (Figure 3-1A). Moreover, Skp2 knockdown in multiple cancer cell lines (Hep3B, BT-474, and MCF-7) impaired glucose deprivation-induced phosphorylation of ACC (Figures 3-1B–D). Similarly, ACC phosphorylation induced by 2-DG was compromised by Skp2 knockdown (Figure 3-1E). These results indicate that Skp2 is critical for activating the LKB1/AMPK signaling.

We next examined whether Skp2 is critical for maintaining LKB1 kinase activity by measuring LKB1 kinase activity toward AMPK α *in vitro*. Strikingly, LKB1 proteins isolated from cells with Skp2 knockdown displayed a decreased ability to phosphorylate AMPK α , compared with LKB1 derived from cells with control knockdown (Figure 3-2). This indicates that Skp2 deficiency impairs the ability of LKB1 to activate AMPK, thereby downregulating the LKB1/AMPK signaling. Our results suggest that Skp2 is a novel LKB1 regulator that is required for maintaining LKB1 activity.

A



B

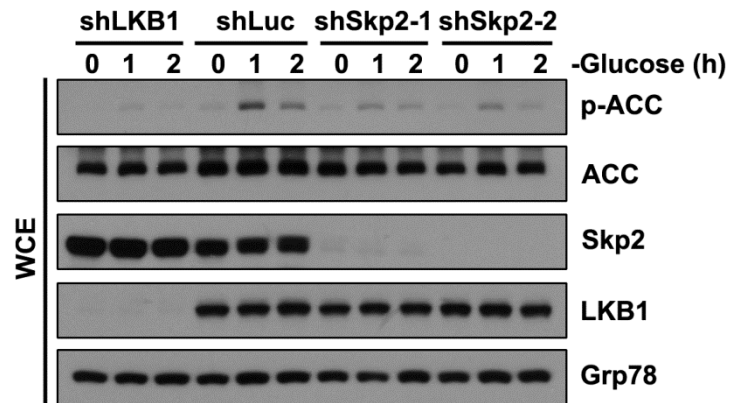
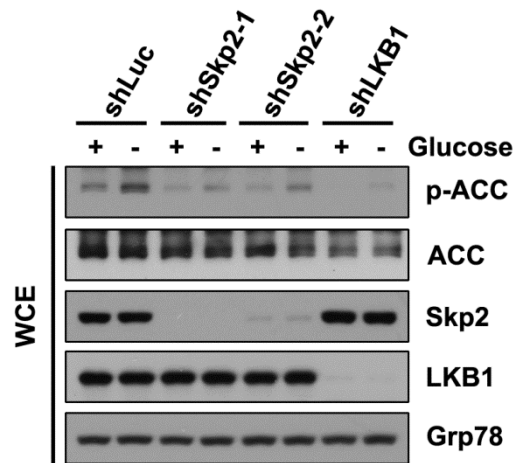


Figure 3-1. The LKB1-AMPK signaling is downregulated under *Skp2* deficiency.

(A) *WT* (*Skp2*^{+/+}) and *Skp2*-KO (*Skp2*^{-/-}) MEFs cultured in the presence (+) or absence (-) of glucose were subjected to immunoblotting.

(B) Hep3B cells with control (shLuc), *Skp2* or LKB1 knockdown cultured in the absence of glucose for the indicated time points were subjected to immunoblotting.

C



D

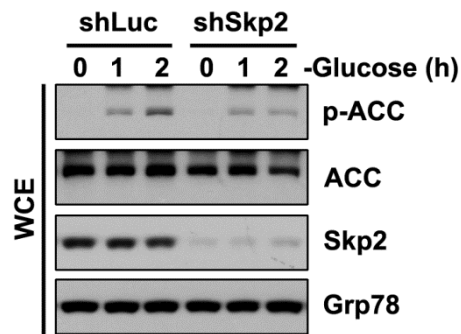


Figure 3-1. The LKB1/AMPK signaling is downregulated under Skp2 deficiency (continued).

(C) BT-474 cells with control (shLuc), Skp2 or LKB1 knockdown cultured in the presence (+) or absence (-) of glucose were subjected to immunoblotting.

(D) MCF-7 cells with control (shLuc) or Skp2 knockdown cultured in the absence of glucose for the indicated time points were subjected to immunoblotting.

E

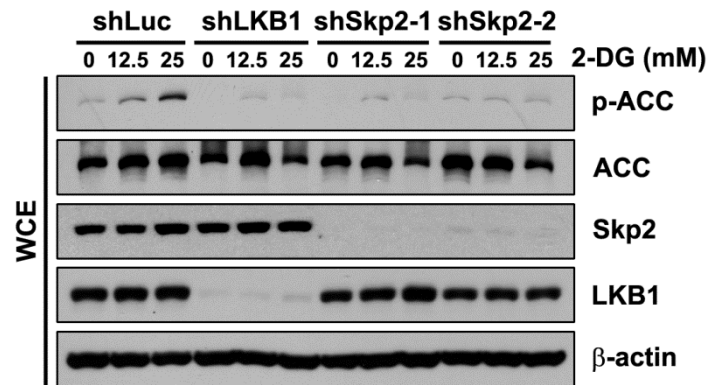


Figure 3-1. The LKB1/AMPK signaling is downregulated under Skp2 deficiency (continued).

(E) HEK293 cells with control (shLuc), Skp2 or LKB1 knockdown after treatment with 2-DG at the indicated concentrations for 15 minutes were subjected to immunoblotting.

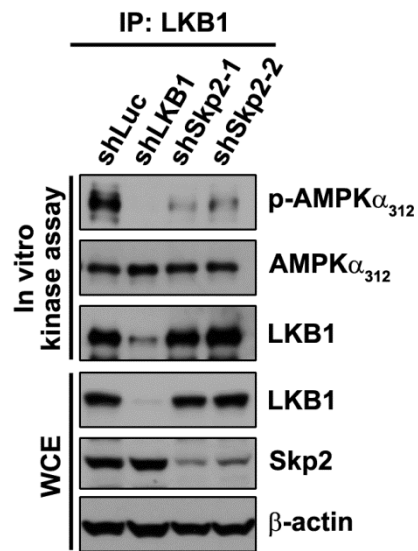


Figure 3-2. Skp2 knockdown attenuates the LKB1 kinase activity.

Immunoprecipitates (IP) by anti-LKB1 antibody from HEK293 cells with control (shLuc), LKB1 or Skp2 knockdown were subjected to *in vitro* LKB1 kinase assay followed by immunoblotting.

3.2. Skp2 Induces K63-linked Polyubiquitination of LKB1

To gain a further insight into how Skp2 regulates LKB1 activity, we examined the interaction between endogenous Skp2 and LKB1 using reciprocal co-immunoprecipitation (co-IP) experiments. We found that Skp2 and LKB1 were co-immunoprecipitated by each other (Figures 3-3A,B). The specificity of their interaction was verified in LKB1 or Skp2 knockdown cells, and both LKB1 knockdown and Skp2 knockdown compromised the Skp2-LKB1 co-IP efficiency (Figures 3-3A,B). Because Skp2 is a substrate recognition component of the SCF ubiquitin ligase complex, our finding that Skp2 interacts with LKB1 raises the question of whether Skp2 can promote LKB1 ubiquitination. Using the *in vivo* ubiquitination assay, we found that overexpression of WT Skp2 promoted LKB1 polyubiquitination in the absence of the proteasome inhibitor MG132 (Figure 3-4A). In contrast, the ubiquitin ligase-defective mutant of Skp2 (Skp2-NES), which does not form the Skp2-SCF complex (Lin et al., 2009), failed to promote LKB1 polyubiquitination, although Skp2-NES still bound to LKB1 as efficiently as WT Skp2 (Figures 3-4A,B). Consistently, administration of a specific Skp2 inhibitor, which impairs the ubiquitin ligase activity of the Skp2-SCF complex by preventing Skp2-Skp1 binding (Chan et al., 2013), diminished Skp2-mediated LKB1 polyubiquitination (Figure 3-5). Moreover, Skp2 knockdown reduced LKB1 polyubiquitination (Figure 3-6). To verify whether Skp2-SCF is a *bona fide* ubiquitin ligase toward LKB1, we performed the *in vitro* ubiquitination assay by incubating recombinant GST-LKB1 with the purified SCF/Flag-Skp2 complex, along with recombinant ubiquitin, E1 and E2 enzymes. The Skp2-SCF was capable of ubiquitinating LKB1 directly *in vitro* (Figure 3-7), confirming that the Skp2-SCF is a direct ubiquitin ligase for LKB1. Similar to genetic inhibition of Skp2, pharmacological inactivation of Skp2 by administration of the Skp2 inhibitor downregulated the LKB1/AMPK signaling as determined by decreased

phosphorylation of ACC in a dose-dependent manner (Figure 3-8). Thus, our data indicate that Skp2 orchestrates LKB1 activity through maintaining polyubiquitination of LKB1.

A recent study demonstrated that the ubiquitin ligase CHIP is involved in LKB1 degradation (Gaude et al., 2012). However, we did not observe significant changes in LKB1 protein levels upon Skp2 knockdown or overexpression in our experiments, so we speculated that Skp2 mediates non-degradative polyubiquitination of LKB1. K48-linked ubiquitin chains are known as a major targeting signal for proteasomal degradation, whereas K63-linked ubiquitin chains have non-proteolytic functions in many cellular processes, such as kinase activation, DNA repair, and protein trafficking (Yang et al., 2010). Accordingly, to confirm that Skp2-mediated polyubiquitination of LKB1 is non-proteolytic, we applied two mutant forms of ubiquitin to our *in vivo* ubiquitination assay: ubiquitin-K48R (His-Ub-K48R) and ubiquitin-K63R (His-Ub-K63R), which exclusively eliminate K48-linked and K63-linked polyubiquitination, respectively. Strikingly, we found that Ub-K63R, but not Ub-K48R, blocked Skp2-mediated LKB1 polyubiquitination (Figure 3-9). Because the linkage specificity of ubiquitin chains is determined by E2 ubiquitin-conjugating enzymes, we also investigated the effect of K63-Ub-specific E2 enzymes Ubc13 and Ubc5C on Skp2-mediated LKB1 polyubiquitination. Knockdown of either Ubc13 or Ubc5C decreased Skp2-mediated LKB1 polyubiquitination (Figure 3-10). Hence, Skp2-mediated LKB1 polyubiquitination primarily occurs through non-degradative K63-linked ubiquitination for non-proteolytic regulation, such as kinase activation, which is consistent with our aforementioned finding that Skp2 affects LKB1 activity rather than stability.

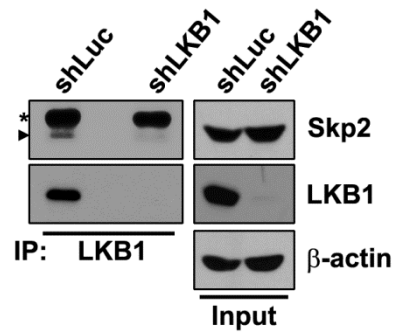
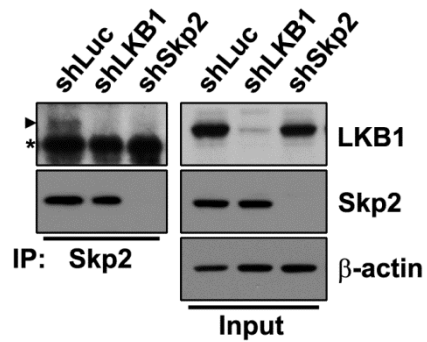
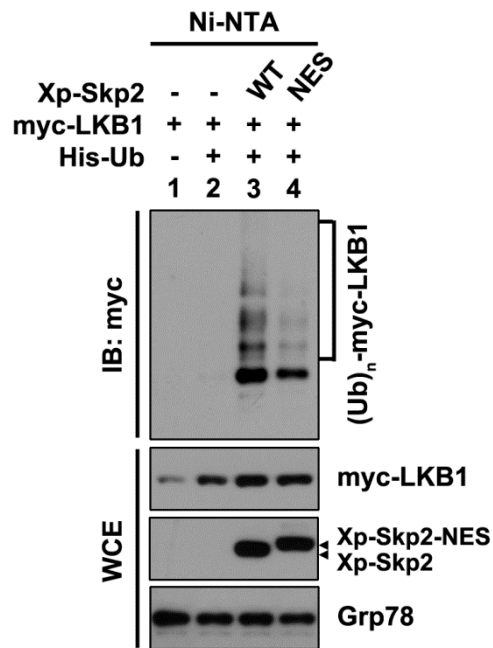
A**B**

Figure 3-3. Skp2 interacts endogenously with LKB1.

(A) (B) Immunoprecipitates by anti-LKB1 **(A)** or anti-Skp2 **(B)** antibody from HEK293 cells with control (shLuc), LKB1 or Skp2 knockdown were subjected to immunoblotting. The asterisks indicate heavy chains of the antibodies and the arrowheads indicate the bands corresponding to Skp2 **(A)** or LKB1 **(B)**.

A



B

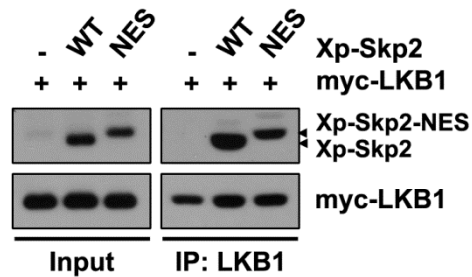


Figure 3-4. Skp2 promotes polyubiquitination of LKB1.

(A) *In vivo* ubiquitination assay in HEK293T cells transfected with the indicated plasmids was followed by immunoblotting.

(B) Immunoprecipitates (IP) by anti-LKB1 antibody from HEK293T cells transfected with the indicated plasmids were subjected to immunoblotting.

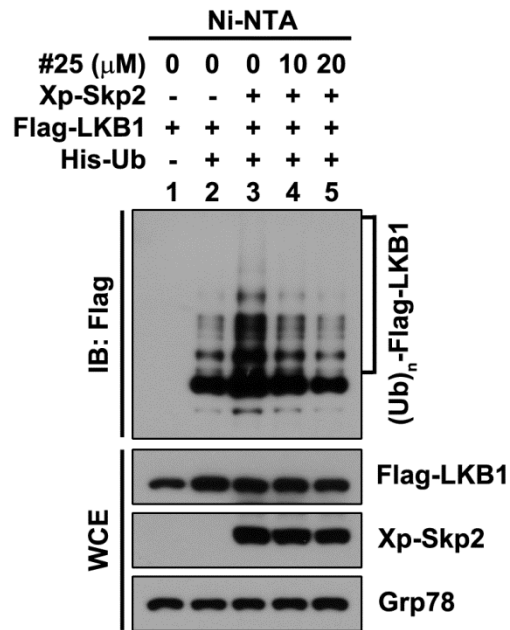


Figure 3-5. Skp2-SCF ubiquitin ligase activity is required for Skp2-mediated polyubiquitination of LKB1.

In vivo ubiquitination assay in HEK293T cells transfected with the indicated plasmids and treated with the Skp2 inhibitor (compound #25) at the indicated concentrations was followed by immunoblotting.

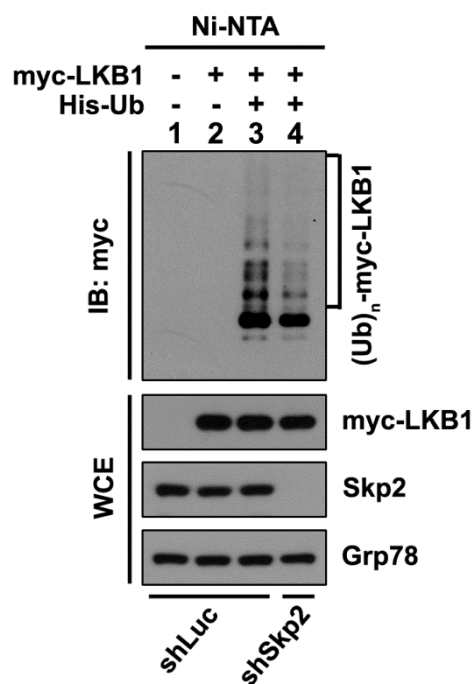


Figure 3-6. Skp2 knockdown attenuates polyubiquitination of LKB1.

In vivo ubiquitination assay in HEK293 cells with control (shLuc) or Skp2 knockdown transfected with the indicated plasmids was followed by immunoblotting.

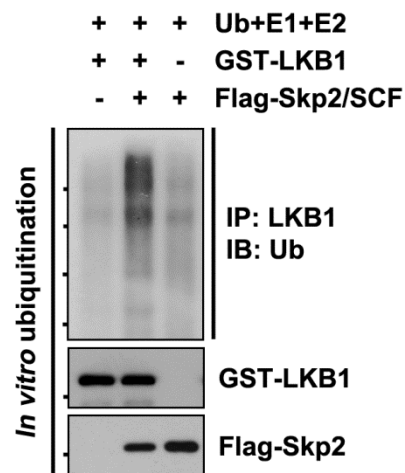


Figure 3-7. Skp2-SCF is capable of ubiquitinating LKB1 *in vitro*.

In vitro ubiquitination assay, in which recombinant GST-LKB1 was incubated with purified Flag-Skp2/SCF, along with recombinant ubiquitin (Ub), E1 and E2 enzymes for reaction, was followed by immunoprecipitation and immunoblotting.

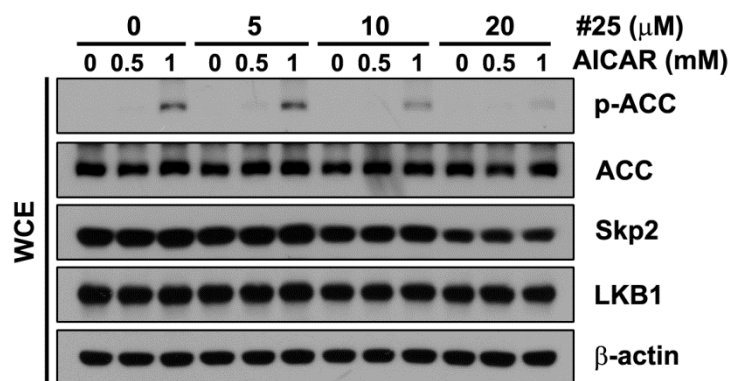


Figure 3-8. Skp2-SCF ubiquitin ligase activity is required for activation of the LKB1/AMPK signaling.

Hep3B cells pretreated with the Skp2 inhibitor (compound #25) at the indicated concentrations for 24 hours were subjected to AICAR treatment at the indicated concentrations for 2 hours, followed by immunoblotting.

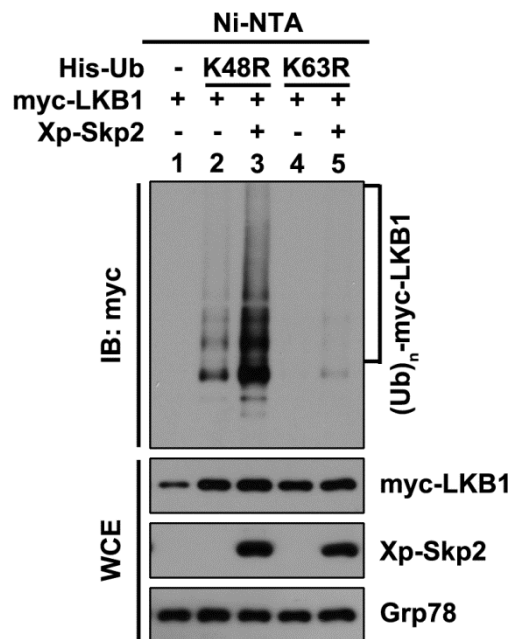


Figure 3-9. Skp2-mediated polyubiquitination of LKB1 primarily occurs via K63-linkage.

In vivo ubiquitination assay in HEK293T cells transfected with the indicated plasmids were followed by immunoblotting.

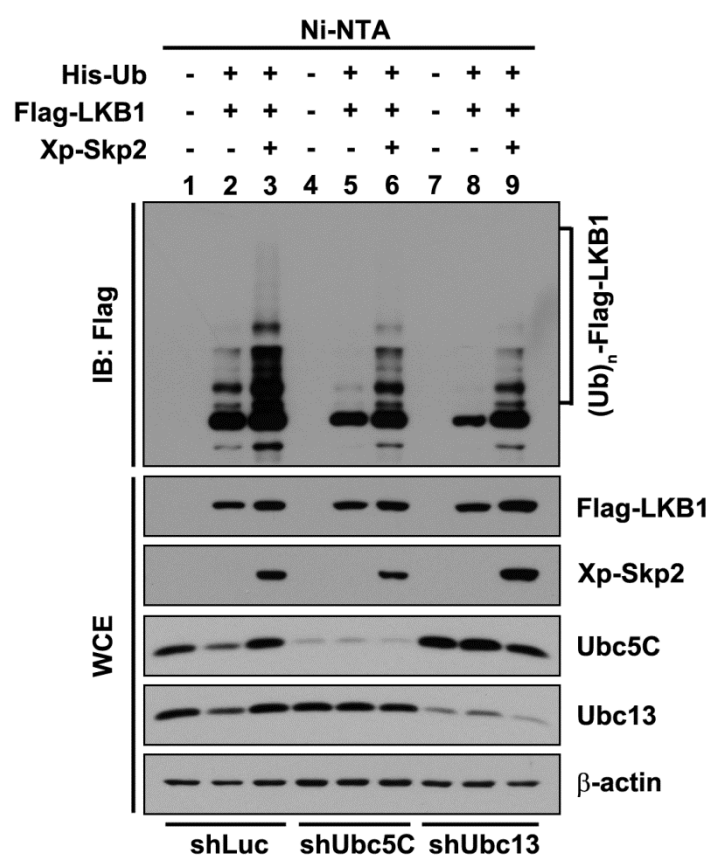


Figure 3-10. K63-specific E2 enzymes are involved in Skp2-mediated polyubiquitination of LKB1.

In vivo ubiquitination assay in HEK293 cells with control (shLuc), Ubc5C or Ubc13 knockdown transfected with the indicated plasmids was followed by immunoblotting.

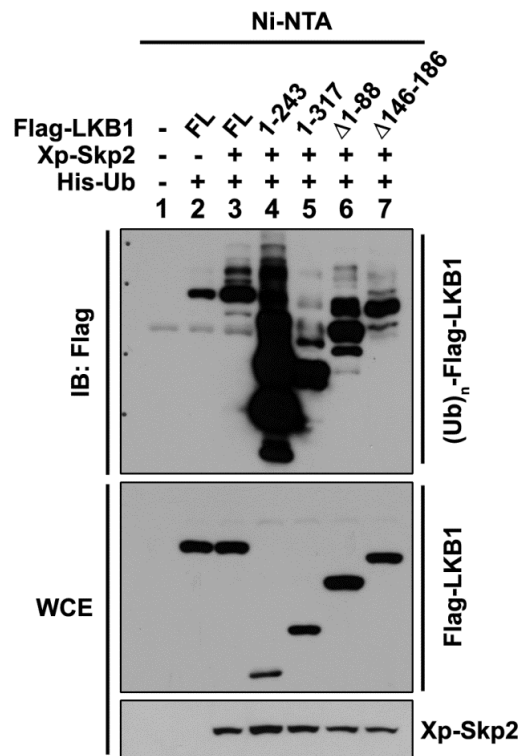
3.3. LKB1 Polyubiquitination Is Critical for LKB1 Activation

To further characterize the role of Skp2-mediated LKB1 polyubiquitination in LKB1 activation, we attempted to identify lysine residue(s) on LKB1 where Skp2-mediated polyubiquitination takes place. Human LKB1 contains 32 lysine residues, and 26 of them are highly conserved among species. We first screened various fragments of LKB1 using *in vivo* ubiquitination assays to locate Skp2-mediated LKB1 polyubiquitination. By comparing polyubiquitination among the LKB1 fragments, we concluded that Skp2-mediated polyubiquitination occurred most intensively within the N-terminus of LKB1 (amino acids 1-243 and 1-87; Figures 3-11A,B and 3-12). Through a series of screening experiments in which mutagenesis of the conserved lysine to arginine (K-to-R) within the region of LKB1 was performed, we found that none of the LKB1 mutants containing a single K-to-R substitution significantly affected LKB1 polyubiquitination (Figures 3-13A,B). By screening LKB1 mutants containing multiple K-to-R substitutions, we identified that the 5KR mutant of LKB1 (LKB1-5KR), which carries 5 K-to-R substitutions at the position 41, 44, 48, 62 and 64, displayed much less Skp2-mediated polyubiquitination as well as basal polyubiquitination than WT LKB1 (Figures 3-13C,D), although LKB1-5KR bound to Skp2 comparably to WT LKB1 (Figure 3-13E). Thus, LKB1-5KR is an ubiquitination-deficient mutant of LKB1.

To understand whether Skp2-mediated K63-linked ubiquitination of LKB1 is important for LKB1 kinase activity, we examined the ability of the ubiquitination-deficient mutant LKB1-5KR to activate the downstream AMPK signaling by ectopic expression of vector, WT LKB1 or LKB1-5KR in LKB1-deficient cells or LKB1-proficient cells with LKB1 knockdown. When stably expressed in LKB1-deficient HeLa cells, WT LKB1, but not kinase-dead (KD) LKB1, strongly activated the downstream AMPK signaling, showing robust induction of ACC phosphorylation compared with the vector control (Figure 3-14A). However, the 5KR mutant

significantly reduced LKB1's ability to induce AMPK signaling, as determined by less phosphorylation of ACC compared with WT LKB1 (Figure 3-14A). Also, under the low-energy conditions—glucose deprivation or 2-DG treatment, LKB1-5KR exhibited a compromised ability to induce ACC phosphorylation in LKB1-deficient A549 cells and *LKB1*-KO MEFs in comparison with WT LKB1 (Figures 3-14B,C). Similarly, LKB1-5KR failed to rescue the defect of LKB1 knockdown in glucose deprivation-induced phosphorylation of ACC in Hep3B cells (Figure 3-14D). To confirm the impact of Skp2-mediated polyubiquitination on LKB1 kinase activity, we determined the kinase activity of LKB1-5KR directly using the *in vitro* kinase assay. Consistently, LKB1-5KR isolated from cells showed decreased kinase activity toward AMPK α compared with WT LKB1 (Figure 3-15). Hence, our data suggest that Skp2-mediated K63-linked ubiquitination of LKB1 serves as a novel regulatory mechanism for LKB1 activation.

A



B

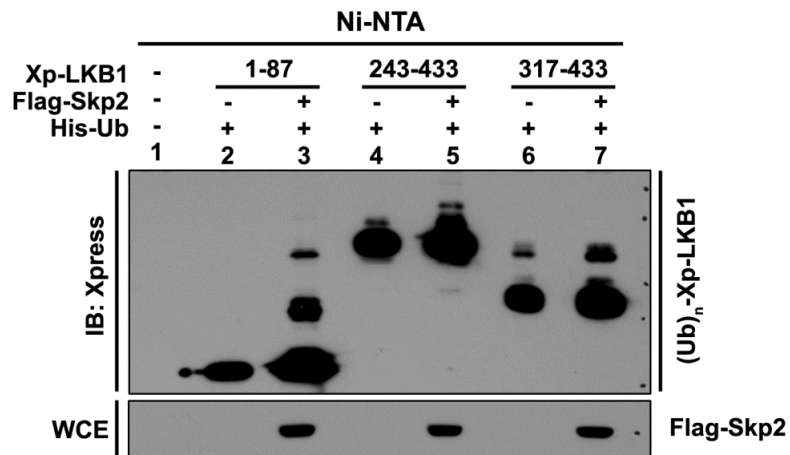


Figure 3-11. Skp2-mediated polyubiquitination primarily occurs within the N-terminus of LKB1.

(A) (B) *In vivo* ubiquitination assays in HEK293T cells transfected with the constructs expressing the indicated fragments of tagged-LKB1 and the other indicated plasmids were followed by immunoblotting.

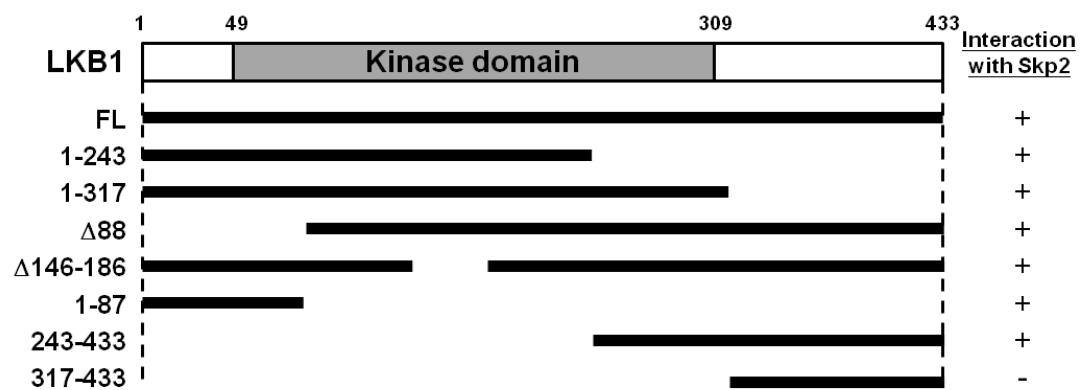


Figure 3-12. A diagram summary of interaction between Skp2 and different fragments of LKB1.

Interaction between the indicated LKB1 fragments and Skp2 was examined by co-immunoprecipitation assays, and the result is presented by a diagram.

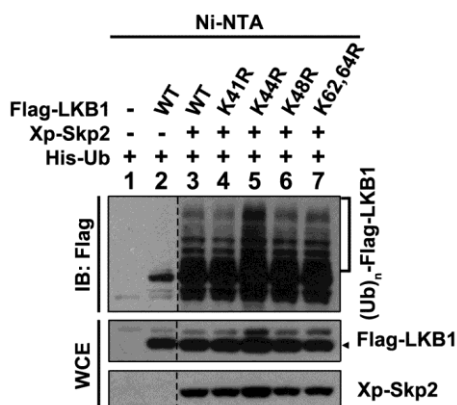
A

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Homo sapiens  -----MEV-VDPQQLGMFTEGELMSVGMDTFIHRIDSTEVIYQPRRKRA
Chimpanzee   -----MEV-VDPQQLGMFTEGELMSVGMDTFIHRIDSTEVIYQPRRKRA
Cow          -----MEV-ADPQQLGMFTEGELMSVGMDTFIHRIDSTEVIYQPRRKRA
Rat          -----MDV-ADPQLGLFPEGELMSVGMDTFIHRIDSTEVIYQPRRKRA
Mouse        -----MDV-ADPEPLGLFSEGELMSVGMDTFIHRIDSTEVIYQPRRKRA
Xenopus      MLCPSMDE-GGSQEIGFLGD--L-SVGMDTFIHRIDSTEVIYQPRRKRA
Zebrafish     -----MSVGADLQHLDYLTENEL--MGMDTFIHRIDSTEVIYQPRRKRA
              * . . . . . * :*****
              44 48      62 64      78 81 84
Homo sapiens  KLIGKYLMDLLGEGSYGKVKVLDSETLCRRAVKILKKKLRRIPNGEA
Chimpanzee   KLIGKYLMDLLGEGSYGKVKVLDSETLCRRAVKILKKKLRRIPNGEA
Cow          KLIGKYLMDLLGEGSYGKVKVLDSETLCRRAVKILKKKLRRIPNGEA
Rat          KLIGKYLMDLLGEGSYGKVKVLDSETLCRRAVKILKKKLRRIPNGEA
Mouse        KLIGKYLMDLLGEGSYGKVKVLDSETLCRRAVKILKKKLRRIPNGEA
Xenopus      KLVGKYLMDLLGEGSYGKVKVLDSETLCRRAVKILKKKLRRIPNGEA
Zebrafish     KLIGKYLMDLLGEGSYGKVKVLDSETLCRRAVKILKKKLRRIPNGEA
              ** :*****

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B



C

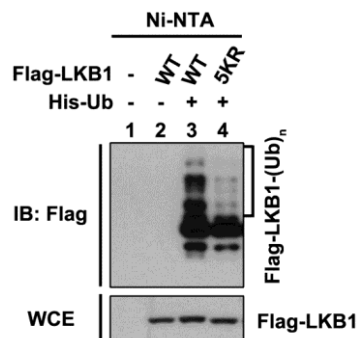
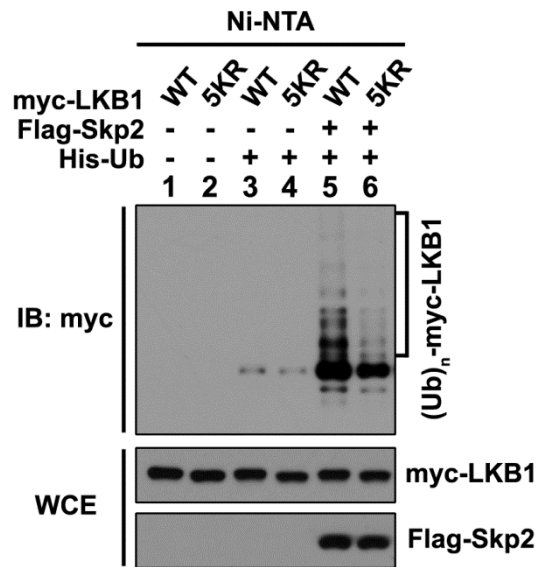


Figure 3-13. The 5KR mutant of LKB1 is an ubiquitination-deficient mutant.

(A) LKB1 protein sequences among species (from Ensembl) were aligned through T-Coffee website (<http://www.ebi.ac.uk/Tools/msa/tcoffee/>). The conserved Lys (K) residues within the N-terminus of LKB1 (1-93 amino acids) are highlighted and the ones corresponding to the 5KR mutation are highlighted in yellow. Lys78 (shown in red) is located in the ATP-binding site of the LKB1 kinase domain.

(B) (C) *In vivo* ubiquitination assays in HEK293T cells transfected with the constructs expressing the indicated mutant forms of Flag-LKB1 and the other indicated plasmids were followed by immunoblotting.

D



E

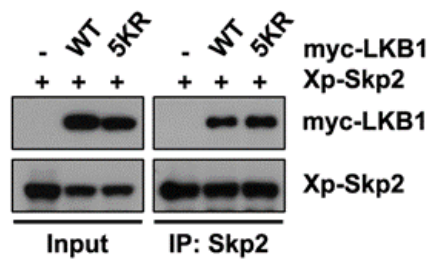


Figure 3-13. The 5KR mutant of LKB1 is an ubiquitination-deficient mutant (continued).

(D) *In vivo* ubiquitination assay in HEK293T cells transfected with the indicated plasmids were followed by immunoblotting.

(E) Immunoprecipitates (IP) by anti-Skp2 antibody from HEK293T cells transfected with the indicated plasmids were subjected to immunoblotting.

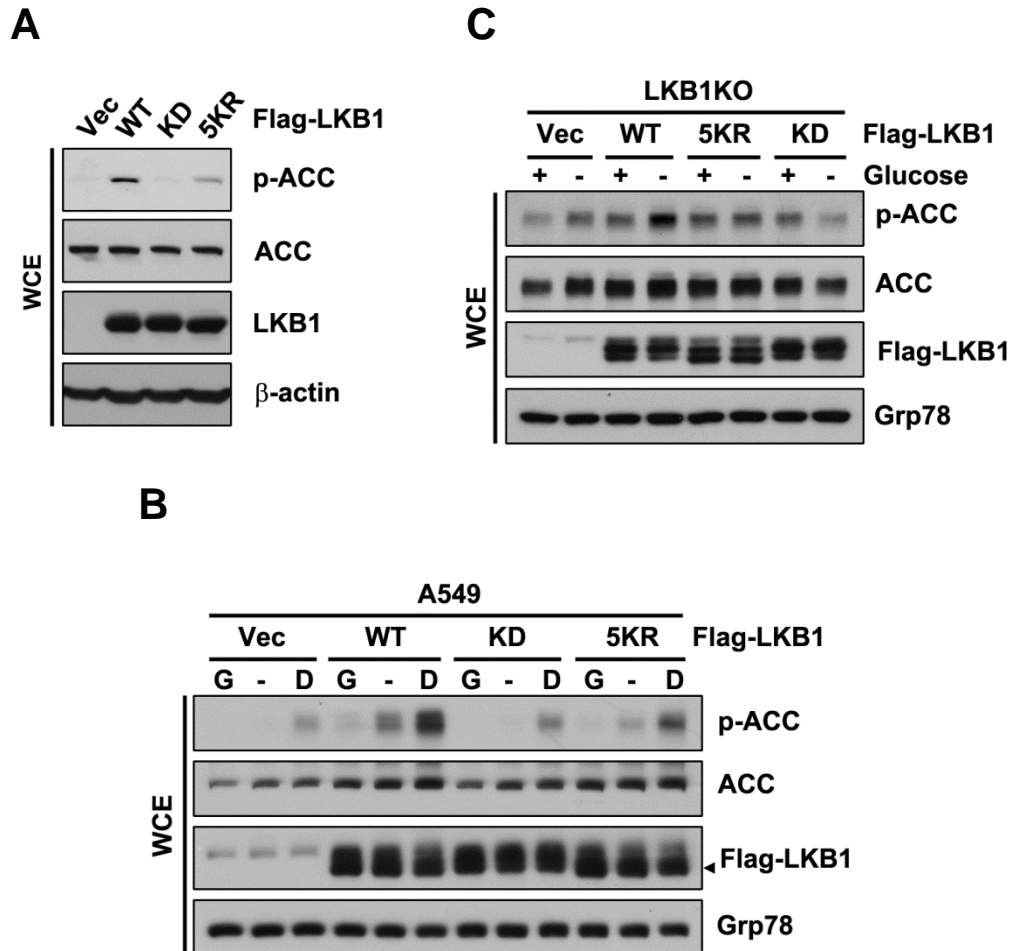


Figure 3-14. Skp2-mediated polyubiquitination of LKB1 is important for activation of the LKB1/AMPK signaling.

(A) LKB1-deficient HeLa cells with stable restoration of the indicated Flag-LKB1 were subjected to immunoblotting.

(B) *LKB1*-deficient A549 cells with stable reconstitution of the indicated Flag-LKB1 cultured with (G) or without (-) glucose, or treated with 5mM 2-DG (D) for 2 hours were subjected to immunoblotting.

(C) *LKB1*-KO MEFs with stable reconstitution of the indicated Flag-LKB1 cultured in the presence (+) or absence (-) of glucose for 2 hours were subjected to immunoblotting.

D

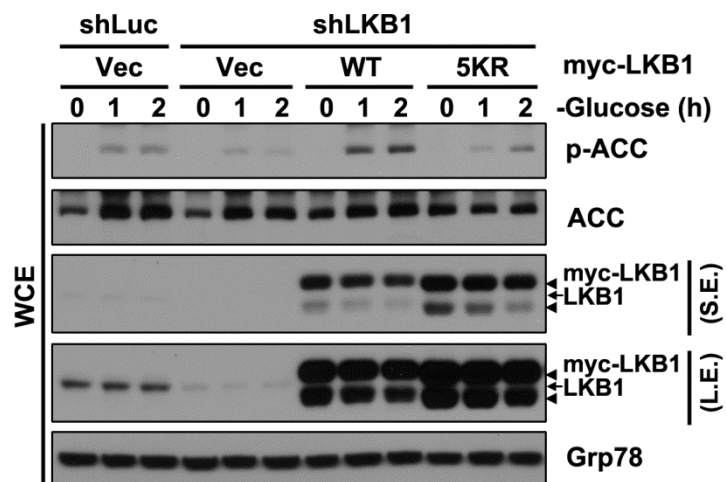


Figure 3-14. Skp2-mediated polyubiquitination of LKB1 is important for activation of the LKB1/AMPK signaling (continued).

(D) Control- or LKB1-knockdown Hep3B cells with stable transduction of the indicated shRNA non-targeting myc-LKB1 cultured in the absence of glucose for the indicated time points were subjected to immunoblotting. S.E., short exposure; L.E., long exposure.

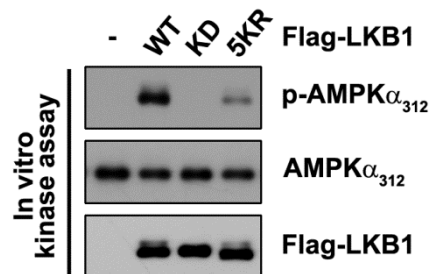


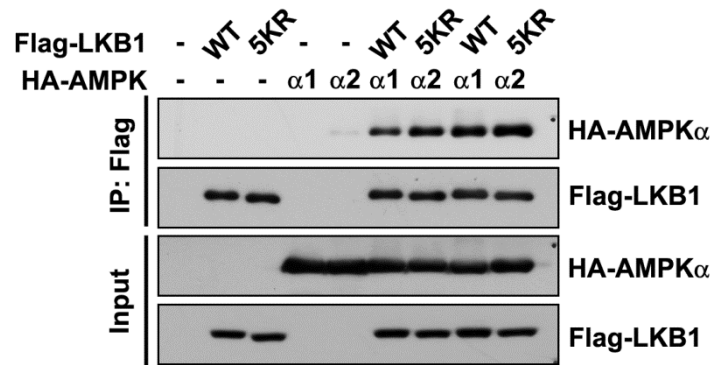
Figure 3-15. Skp2-mediated polyubiquitination of LKB1 is critical for LKB1 kinase activity.

Immunoprecipitates of exogenous Flag-LKB1 from HEK293T cells transfected with the indicated Flag-LKB1 were subjected to *in vitro* LKB1 kinase assay followed by immunoblotting.

3.4. LKB1 Polyubiquitination Is Crucial for Maintaining LKB1 Complex Integrity

Next, we sought to determine the molecular mechanism of how Skp2-mediated K63-linked ubiquitination of LKB1 regulates LKB1 activity. First, we examined the ability of LKB1 to bind to its substrate AMPK α . Binding of LKB1 to AMPK α 1/2 was not affected by either LKB1-5KR mutant or Skp2 knockdown in the co-IP experiments (Figures 3-16A,B). Considering that LKB1 is mainly activated through the formation of the LKB1-STRAD-MO25 complex, we then investigated the integrity of the LKB1 heterotrimeric complex using co-IP experiments. In the co-IP experiment, there was no significant difference in the LKB1-STRAD interaction between WT LKB1 and LKB1-5KR; however, the ubiquitination-deficient mutant LKB1-5KR displayed impaired ability to bind to the other subunit MO25 (Figure 3-17A). Attenuated interaction between LKB1 and MO25 was also observed in multiple LKB1-deficient cell lines (HeLa, A549, and *LKB1*-KO MEFs) reconstituted with LKB1-5KR (Figures 3-17B–D). Furthermore, Skp2 knockdown, which attenuated LKB1 polyubiquitination, impaired the LKB1-MO25 interaction (Figure 3-17E). Since STRAD, but not MO25, has been demonstrated to facilitate nucleocytoplasmic shuttling of LKB1 (Dorfman and Macara, 2008), our result that LKB1 ubiquitination does not influence the LKB1-STRAD binding could explain our own observation that Skp2-mediated polyubiquitination of LKB1 does not affect the nucleocytoplasmic localization of LKB1 (Figures 3-18A–C). Taken together, Skp2-mediated K63-linked polyubiquitination of LKB1 modulates LKB1 kinase activity by maintaining the integrity of the LKB1 complex.

A



B

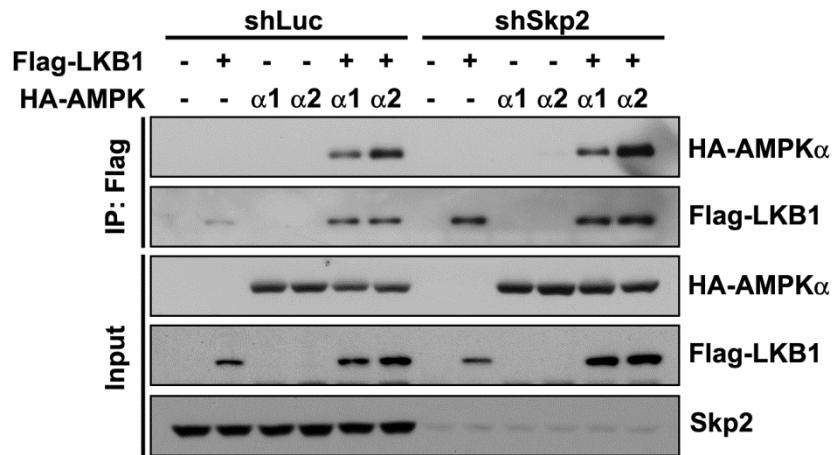


Figure 3-16. Skp2-mediated polyubiquitination of LKB1 does not affect LKB1 binding to AMPK α .

(A) HEK293T cells transfected with the indicated Flag-LKB1 and HA-AMPK α constructs were subjected to immunoprecipitation (IP) followed by immunoblotting.

(B) HEK293 cells with control (shLuc) or Skp2 knockdown transfected with Flag-LKB1 and the indicated HA-AMPK α constructs were subjected to immunoprecipitation followed by immunoblotting.

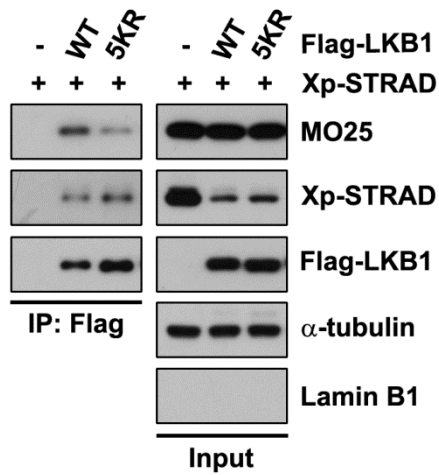
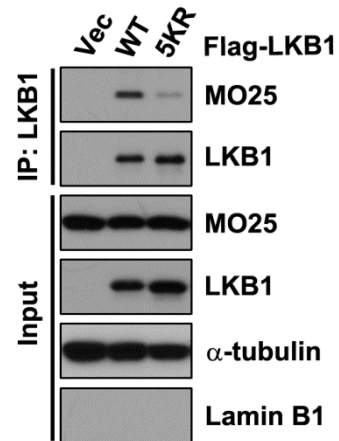
A**B**

Figure 3-17. Skp2-mediated polyubiquitination of LKB1 regulates the integrity of the LKB1 complex.

(A) Immunoprecipitates by anti-Flag antibody from cytosolic fractions of HEK293T cells transfected with Xp-STRAD and the indicated Flag-LKB1 were subjected to immunoblotting.

(B) Immunoprecipitates by anti-LKB1 antibody from cytosolic fractions of LKB1-deficient HeLa cells with stable reconstitution of the indicated Flag-LKB1 were subjected to immunoblotting.

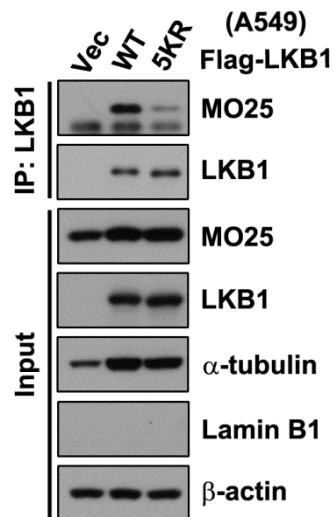
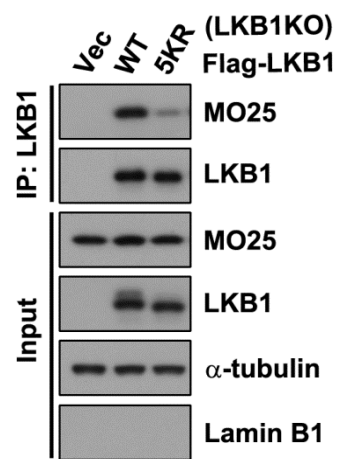
C**D**

Figure 3-17. Skp2-mediated polyubiquitination of LKB1 regulates the integrity of the LKB1 complex (continued).

(C) (D) Immunoprecipitates by anti-LKB1 antibody from cytosolic fractions of LKB1-deficient A549 cells **(C)** or *LKB1*-KO MEFs **(D)** with stable reconstitution of the indicated Flag-LKB1 were subjected to immunoblotting.

E

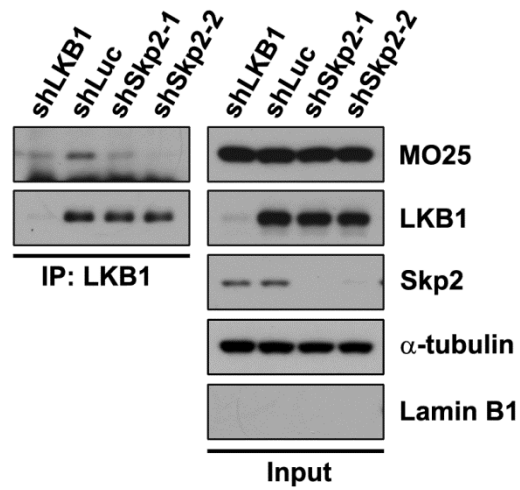


Figure 3-17. Skp2-mediated polyubiquitination of LKB1 regulates the integrity of the LKB1 complex (continued).

(E) Immunoprecipitates by anti-LKB1 antibody from cytosolic fractions of BT-474 cells with control (shLuc), Skp2 or LKB1 knockdown were subjected immunoblotting.

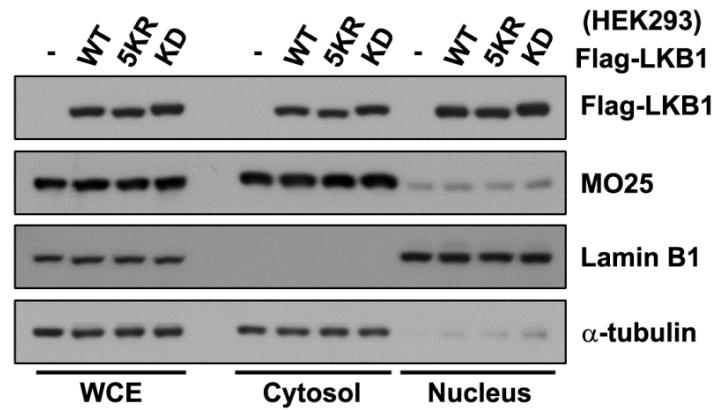
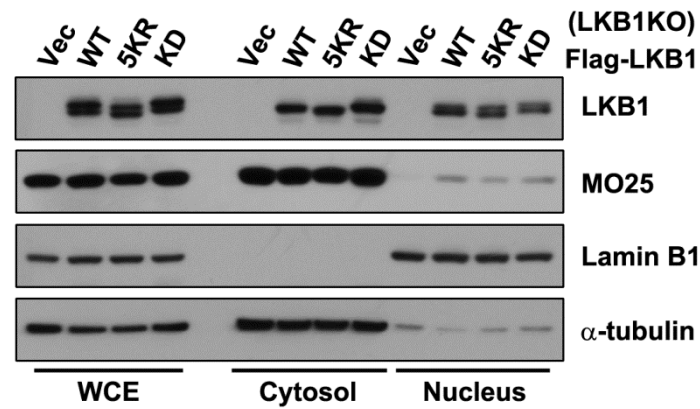
A**B**

Figure 3-18. Skp2-mediated polyubiquitination of LKB1 does not affect LKB1 subcellular localization.

(A) (B) HEK293 cells transfected with the indicated Flag-LKB1 **(A)** and *LKB1*-KO MEFs with stable reconstitution of the indicated Flag-LKB1 **(B)** were subjected to nuclear/cytoplasmic fractionation followed by immunoblotting. Lamin B1 serves as a nuclear marker, and α -tubulin serves as a cytoplasmic marker. WCE, whole cell extracts.

C

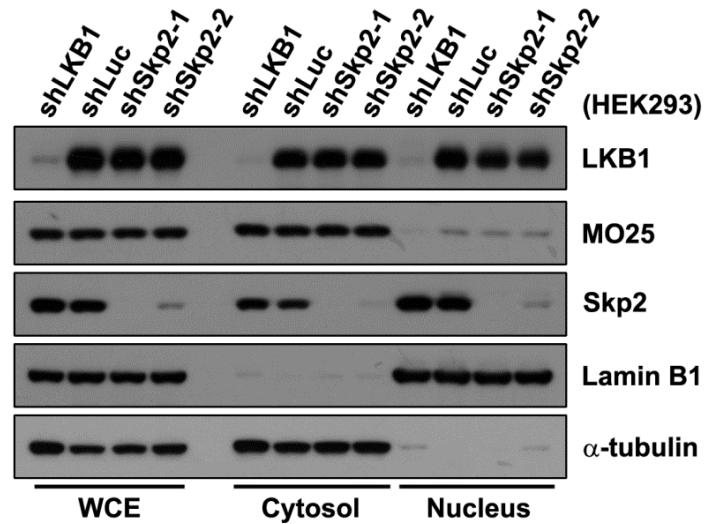


Figure 3-18. Skp2-mediated polyubiquitination of LKB1 does not affect LKB1 subcellular localization (continued).

(C) HEK293 cells with control (shLuc), LKB1 or Skp2 knockdown were subjected to nuclear/cytoplasmic fractionation followed by immunoblotting. Lamin B1 serves as a nuclear marker, and α -tubulin serves as a cytoplasmic marker. WCE, whole cell extracts.

3.5. Ras Activates the Skp2-SCF to Induce LKB1 Polyubiquitination and Activation

We further attempted to identify the upstream regulator(s) that can induce Skp2-mediated polyubiquitination and activation of LKB1. Surprisingly, we found that hyperactivation of Ras by overexpression of constitutively active Ras (H-Ras^{V12}) promoted polyubiquitination of exogenous and endogenous LKB1 (Figures 3-19A,B), which occurred mainly via K63-linked polyubiquitination (Figure 3-20). Here, phosphorylation of Erk1/2 at Thr202/Tyr204 was examined as an indicator of Ras activation. To further assess whether Ras-induced LKB1 polyubiquitination is dependent on Skp2, the Skp2 inhibitor and the ubiquitination-deficient mutant of LKB1 (LKB1-5KR) were used in the *in vivo* ubiquitination assays. In the presence of the Skp2 inhibitor, where Skp2-SCF E3 ligase activity is inhibited (Chan et al., 2013), Ras no longer promoted LKB1 polyubiquitination efficiently (Figure 3-21A). A similar defect in Ras-mediated LKB1 polyubiquitination was also observed on the LKB1-5KR mutant (Figure 3-21B). In contrast, when co-overexpressed with Skp2, Ras displayed a robust synergistic effect on polyubiquitination of endogenous LKB1 (Figure 3-22A). Notably, overexpression of constitutively active Ras promoted the assembly of the Skp2-SCF ubiquitin ligase complex (Figure 3-22B; the Skp2-Skp1-Cul1 interaction), indicating that Ras can induce Skp2-SCF E3 ligase activity.

We next investigated whether hyperactivation of Ras has effect on LKB1 activation. We found that overexpression of constitutively active Ras upregulated the LKB1/AMPK signaling as indicated by increased phosphorylation of ACC and Raptor (at Ser792), two well-established AMPK substrates (Figure 3-23A). We then determined whether Ras-mediated activation of the LKB1/AMPK signaling involves Skp2-dependent LKB1 ubiquitination. Skp2 knockdown as well as LKB1 knockdown diminished Ras-mediated activation of the LKB1/AMPK signaling, showing reduced

induction of ACC and Raptor phosphorylation in comparison with control knockdown (Figure 3-23B), and Ras co-overexpressed with WT LKB1, but not LKB1-5KR or LKB1-KD, further induced the LKB1/AMPK signaling (Figures 3-23C,D). Thus, our results suggest that oncogenic Ras induces K63-linked polyubiquitination of LKB1 and activation of the LKB1/AMPK signaling via activation of the Skp2-SCF ubiquitin ligase complex.

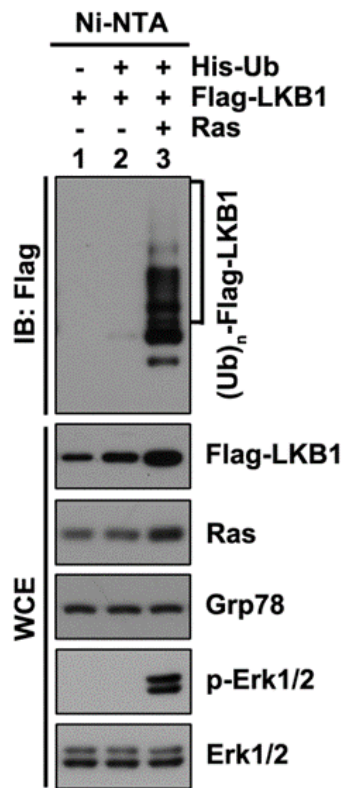
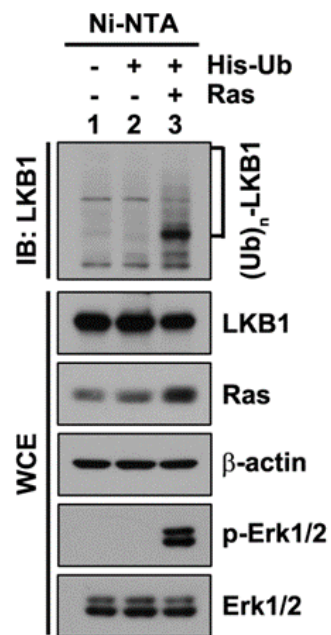
A**B**

Figure 3-19. Overexpression of oncogenic Ras promotes polyubiquitination of LKB1.

(A) (B) *In vivo* ubiquitination assays in HEK293T cells transfected with the indicated plasmids were followed by immunoblotting.

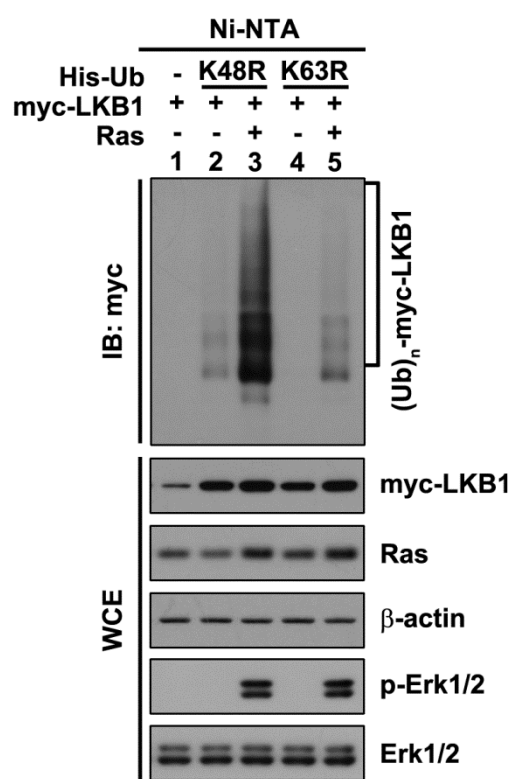


Figure 3-20. Ras-mediated polyubiquitination of LKB1 is mainly through K63-linkage.

In vivo ubiquitination assay in HEK293T cells transfected with the indicated plasmids was followed by immunoblotting.

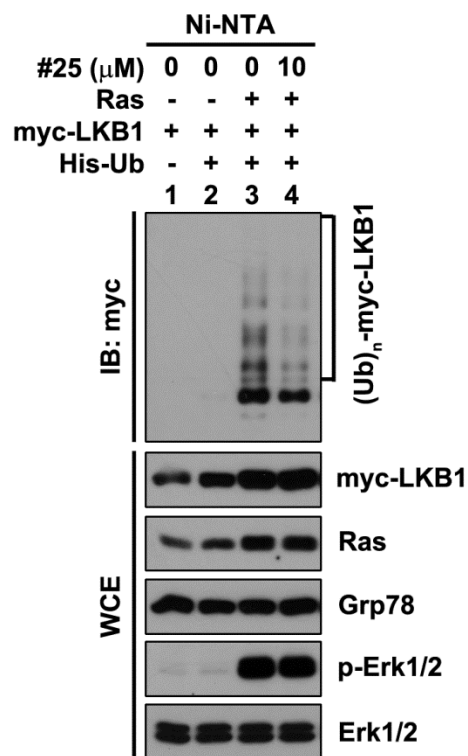
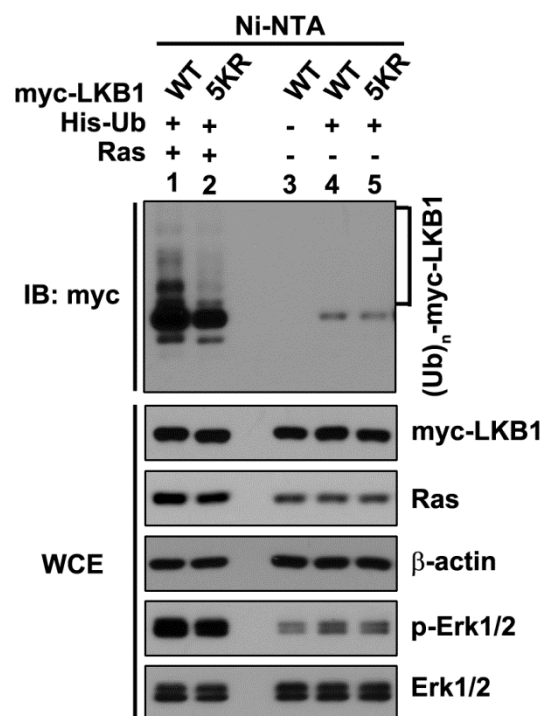
A**B**

Figure 3-21. Oncogenic Ras induces polyubiquitination of LKB1 in a Skp2-SCF-dependent manner.

(A) *In vivo* ubiquitination assay in HEK293T cells transfected with the indicated plasmids and treated with vehicle or the Skp2 inhibitor (compound #25) was followed by immunoblotting.

(B) *In vivo* ubiquitination assay in HEK293T cells transfected with the indicated plasmids was followed by immunoblotting.

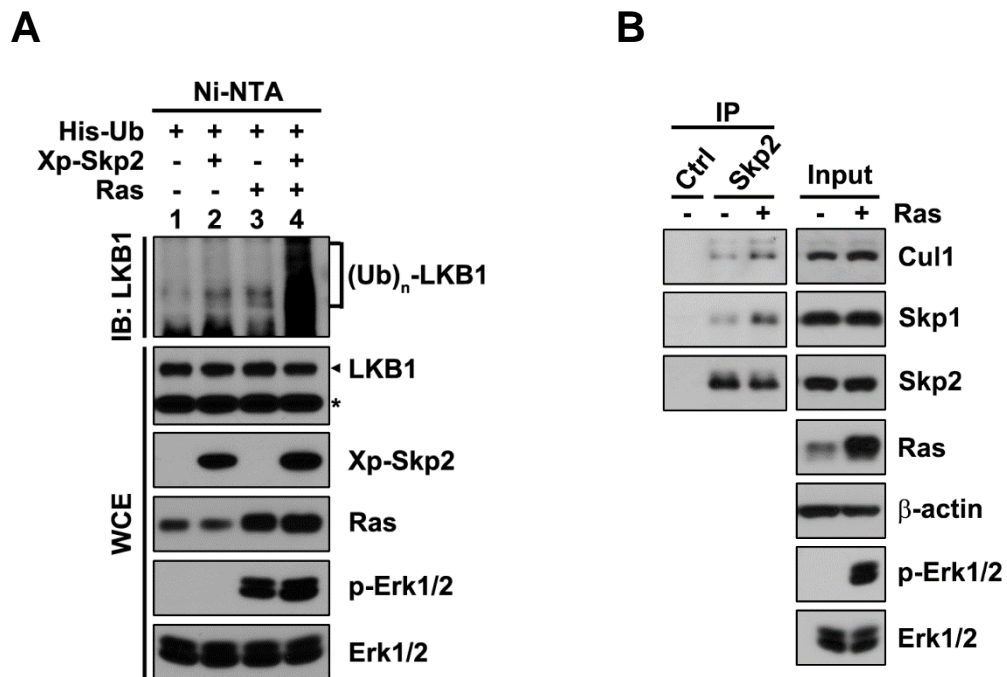


Figure 3-22. Oncogenic Ras activates Skp2-SCF to synergistically promote polyubiquitination of LKB1.

(A) *In vivo* ubiquitination assay in HEK293T cells transfected with the indicated plasmids was followed by immunoblotting. The asterisk indicates non-specific bands (serving as loading controls), and the arrowhead indicates the bands corresponding to LKB1.

(B) Immunoprecipitates (IP) by control or anti-Skp2 antibody from HEK293T cells transfected with vector (Vec) or Ras were subjected to immunoblotting.

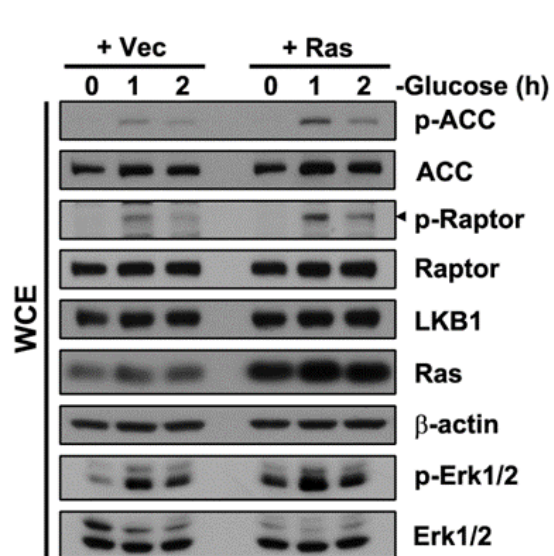
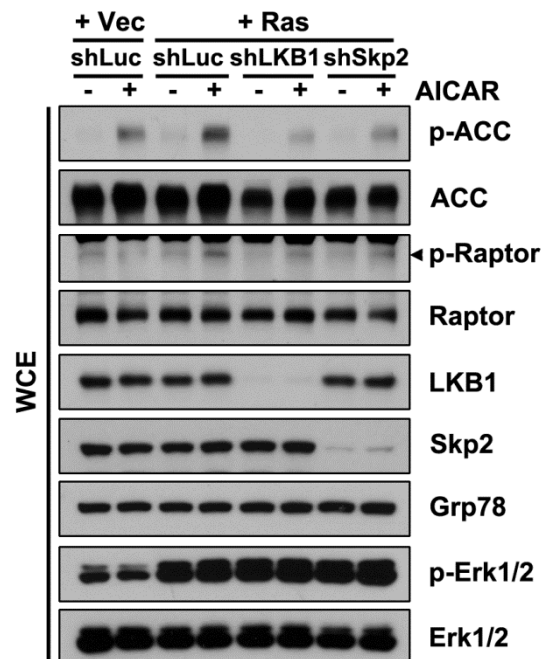
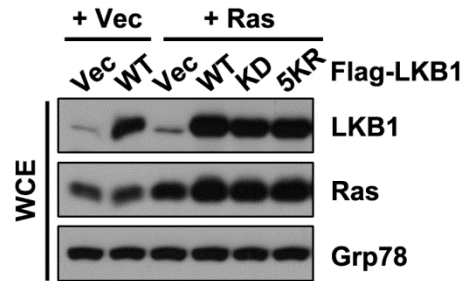
A**B**

Figure 3-23. Oncogenic Ras induces activation of the LKB1/AMPK signaling via Skp2.

(A) WT MEFs with stable transduction of vector (Vec) or Ras cultured in the absence of glucose for the indicated time points were subjected to immunoblotting.

(B) Hep3B cells with stable transduction of vector (Vec) or Ras along with the indicated stable knockdown were subjected to treatment of 1mM AICAR for 2 hours, followed by immunoblotting.

C



D

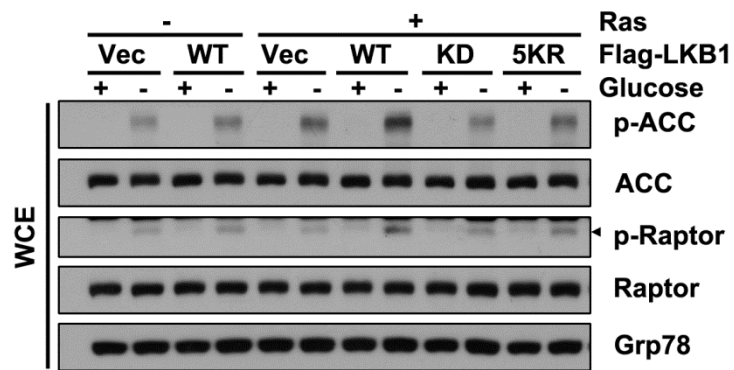


Figure 3-23. Oncogenic Ras induces activation of the LKB1/AMPK signaling via Skp2 (continued).

(C) Hep3B cells with stable transduction of vector (Vec) or Ras along with the indicated Flag-LKB1 were subjected to immunoblotting.

(D) Hep3B cells as in **(C)** cultured in the presence (+) or absence (-) of glucose were subjected to immunoblotting.

3.6. LKB1 Polyubiquitination Regulates LKB1's Function in Cell Survival

As kinase activity has been shown to be critical for LKB1 to execute its biological function via the LKB1/AMPK axis during energy stress, we therefore investigated whether Skp2-mediated K63-linked polyubiquitination of LKB1 regulates LKB1 function in energy stress responses. Activation of the LKB1/AMPK pathway plays an important role in protecting cells from apoptosis under metabolic stress. Accordingly, the effect of LKB1-5KR on energy stress-induced cell survival was examined. As demonstrated in previous studies (Shaw et al., 2004b), compared with the vector control, WT LKB1 exhibited a protective effect on cell viability in LKB1-deficient cells under metabolic stress induced by glucose deprivation (Figure 3-24A) or treatment of AMP analogue AICAR (Figure 3-24B). In contrast, LKB1-5KR, which had a compromised kinase activity, failed to protect LKB1-deficient cells from metabolic stress-induced cell death compared to WT LKB1 (Figures 3-24A,B).

Several evidence has suggested that autophagy, a process that degrades damaged proteins and organelles in response to low nutrient availability to maintain energy homeostasis, is activated by AMPK through activation of ULK1 and inhibition of mTORC1 to serve as a survival/adaptation mechanism under nutrient deprivation (Egan et al., 2011; Kim et al., 2011). Moreover, recent studies demonstrated that the AMPK/ULK1 signaling and LKB1 have function in mitophagy, a selective form of autophagy that degrades damaged mitochondria, under metabolic stress (Egan et al., 2011; Shackelford et al., 2013). We then assessed the role of LKB1 polyubiquitination in mitochondrial homeostasis. Glucose deprivation caused increases in dysfunctional mitochondria, as assayed by flow cytometry following co-staining of cells with MitoTracker Green and MitoTracker Red (Figures 3-25A,B). Notably, ectopic expression of WT LKB1, but not LKB1-5KR, led to a reduced number of defective mitochondria under glucose deprivation (Figures 3-25A,B). Similar results were

obtained in *Skp2*-KO MEFs, showing that *Skp2* deficiency, which lacks a functional LKB1/AMPK signaling, resulted in elevated cell death (Figure 3-26A) and accumulation of defective mitochondria (Figure 3-26B) under metabolic stress. These results show that *Skp2*-mediated K63-linked polyubiquitination of LKB1 is critical for LKB1 function in cell survival during energy stress.

Given our above finding that Ras hyperactivation induces *Skp2*-dependent polyubiquitination and activation of LKB1, we reasoned that Ras may activate the LKB1/AMPK pathway to protect cancer cells against metabolic stress-induced cell death. In line with this notion, we observed that Ras hyperactivation reduced glucose deprivation-induced cell death (Figure 3-27). However, either LKB1 knockdown or *Skp2* knockdown abolished Ras-mediated protective effect on cell survival under glucose starvation (Figure 3-27). Our results underscore the critical role of the Ras/*Skp2*/LKB1 axis in regulating cell survival during energy stress.

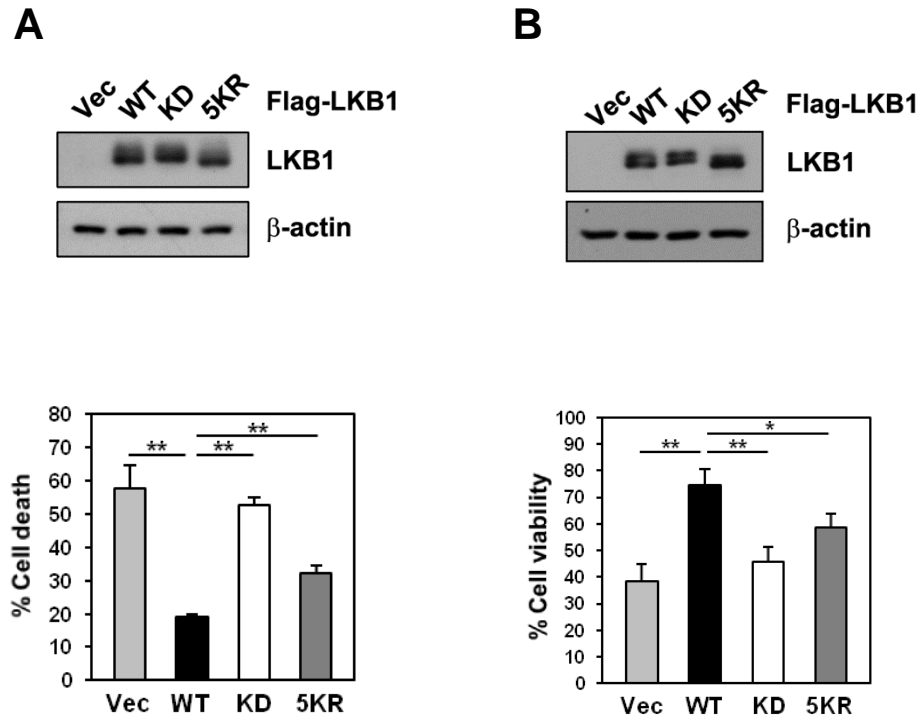


Figure 3-24. Skp2-mediated polyubiquitination of LKB1 is important for cancer cell survival under energy stress.

(A) LKB1-deficient A549 cells stably restored with the indicated Flag-LKB1 were subjected to glucose starvation for 48 hours, and then cell death was determined by DAPI staining. The result is shown as means \pm s.d. ($n \geq 3$). Whole cell extracts of the untreated cells were subjected to immunoblotting (upper panel). ** $P < 0.01$.

(B) LKB1-KO MEFs stably restored with the indicated Flag-LKB1 were subjected to treatment with 2mM AICAR for 24 hours. Cell viability was determined by trypan blue exclusion assay, and is expressed as a percentage of the vehicle-treated controls. The result is shown as means \pm s.d. ($n = 3$). Whole cell extracts of the untreated MEFs were subjected to immunoblotting (upper panel). * $P < 0.05$; ** $P < 0.01$.

A

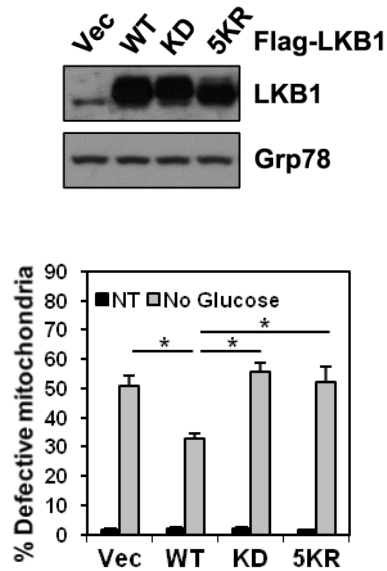


Figure 3-25. Skp2-mediated polyubiquitination of LKB1 is critical for LKB1's function in mitochondrial homeostasis.

(A) Mitochondria of Hep3B cells with stable transduction of the indicated Flag-LKB1 cultured in the presence (NT, non-treated) or absence of glucose for 10 hours were analyzed by flow cytometry with MitoTracker staining. The result is shown as means \pm s.d. ($n=2$). Whole cell extracts of the untreated cells were subjected to immunoblotting (upper panel). * $P<0.05$.

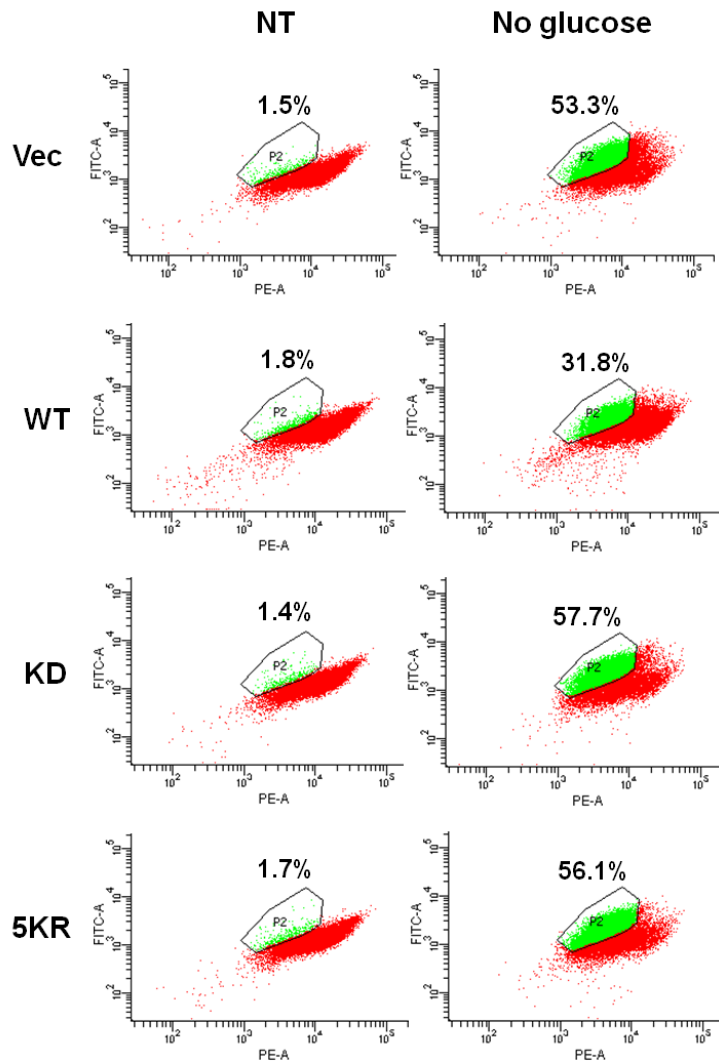
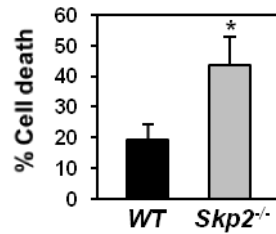
B

Figure 3-25. Skp2-mediated polyubiquitination of LKB1 is critical for LKB1's function in mitochondrial homeostasis (continued).

(B) Representative flow cytometric dot plots from **(A)**. Mitochondria were co-stained with MitoTracker Green (FITC) and MitoTracker Red (PE) followed by flow cytometric analysis. The damaged mitochondria (FITC-positive and PE-negative or low; P2 fraction) were quantitated, and the percentages are shown. NT, non-treated.

A



B

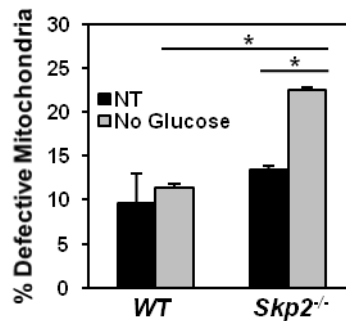


Figure 3-26. Skp2 is involved in energy stress-induced cell survival.

(A) WT and *Skp2*-KO (*Skp2*^{-/-}) MEFs were subjected to treatment with 2mM AICAR for 24 hours. Cell viability was determined by trypan blue exclusion assay, and cell death is expressed as a percentage of the untreated controls. The result is shown as means \pm s.d. ($n=3$). * $P<0.05$.

(B) Mitochondria of WT and *Skp2*-KO (*Skp2*^{-/-}) MEFs cultured in the presence (NT) or absence of glucose for 15 hours were analyzed by flow cytometry following MitoTracker staining. The result is shown as means \pm s.d. ($n=2$). * $P<0.05$.

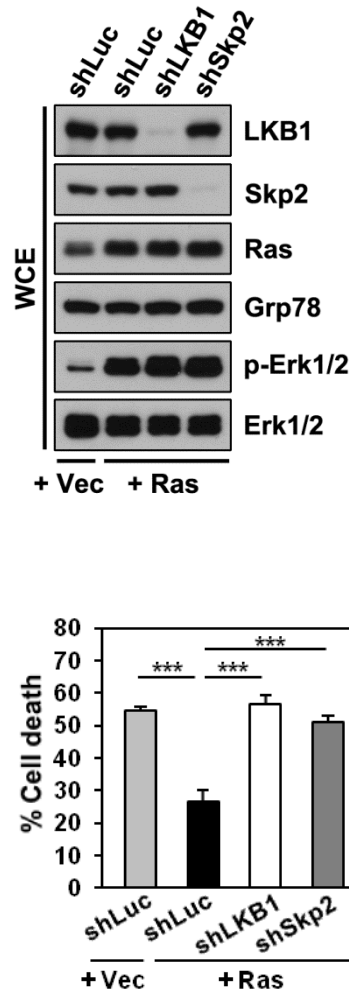


Figure 3-27. The Ras/Skp2/LKB1 axis is important for cancer cell survival under energy stress.

Hep3B cells with stable transduction of vector (Vec) or Ras along with the indicated stable knockdown were subjected to glucose starvation for 8 hours, and then cell death was determined by DAPI staining. The result is shown as means \pm s.d. ($n=4$). Whole cell extracts of the untreated cells were subjected to immunoblotting (upper panel). *** $P<0.005$.

3.7. LKB1 Is Overexpressed and Oncogenic in HCC

Skp2 displays oncogenic activity *in vitro* and *in vivo*, and overexpression of Skp2 has been reported in a variety of human cancers (Chan et al., 2010b), including HCC (Calvisi et al., 2009; Lu et al., 2009). Our findings that Skp2 triggers K63-linked polyubiquitination and activation of LKB1 raise the possibility that LKB1 may also exhibit oncogenic activity in certain tissues like the liver. To test this idea, we therefore assessed the expression of Skp2 and LKB1 and their correlation in human HCC patients. First, we analyzed the expression-profiling dataset of HCC tissues versus normal liver tissues from Gene Expression Omnibus, and found that both *LKB1* (*STK11*) and *Skp2* transcripts were upregulated and correlated in HCC (Figure 3-28A). Consistently, in our HCC patient cohorts, we detected that both Skp2 and LKB1 were overexpressed in late-stage HCC (Figure 3-28B). Our result is in agreement with the previous report showing that LKB1 is overexpressed in human HCC (Martinez-Lopez et al., 2012). In our patient cohorts, the increased expression of both Skp2 and LKB1 was significantly associated with numerous adverse clinical features, including a worse Pugh-Child's classification, the presence of tumor multiplicity, and higher American Joint Committee on Cancer TNM system stage (Table 3-1). Notably, Skp2 expression was also significantly related to advance Cancer of the Liver Italian Program score and Okuda stage (Figure 3-28B and Table 3-1) and positively correlated with LKB1 expression (Figure 3-28C). Moreover, univariate survival analysis results reveal that high expression of Skp2 or LKB1 was remarkably correlated with both overall and local recurrence-free survival (Figures 3-29A–D and Table 3-2). In multivariate survival analysis, Skp2 or LKB1 overexpression was independently predictive for worse overall survival (RR=7.059, $P<0.001$ and RR=2.115, $P=0.035$, respectively; Table 3-3), and only LKB1 expression level was prognostically significant for local recurrence-free survival (RR=3.027, $P < 0.001$;

Table 3-3). Thus, our data show that both Skp2 and LKB1 are overexpressed in HCC patients and their overexpression serves as prognostic markers for poor survival outcome.

To further determine whether overexpression of LKB1 is oncogenic in HCC, we stably overexpressed LKB1 in the human HCC cell line Hep3B and then investigated tumorigenicity of the LKB1-overexpressing Hep3B cells *in vivo* by xenograft assay. Overexpression of WT LKB1 promoted HCC tumor growth *in vivo* in a mouse subcutaneous xenograft tumor model, whereas overexpression of KD LKB1 or LKB1-5KR failed to do so (Figure 3-30). Hence, our data suggest that LKB1 displays oncogenic activity in HCC in a manner dependent on its polyubiquitination and kinase activity.

A

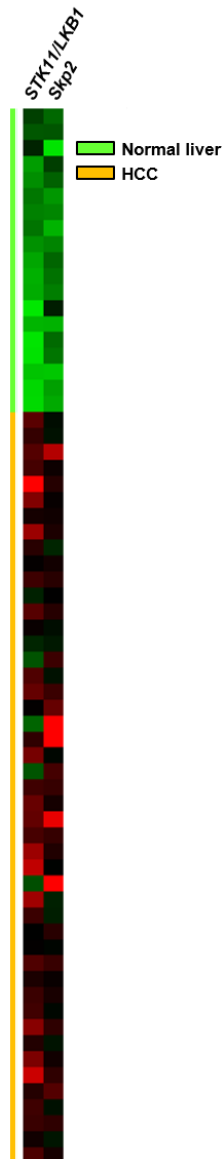


Figure 3-28. Both LKB1 and Skp2 expression are upregulated and correlated in HCC.

(A) The expression-profiling dataset of HCC tissues ($n=47$) versus normal liver tissue ($n=19$) from GSE14323 deposited in the Gene Expression Omnibus was analyzed. Both *LKB1* (*STK11*) and *Skp2* transcripts were significantly upregulated in HCC. The analysis was performed by Dr. Chien-Feng Li.

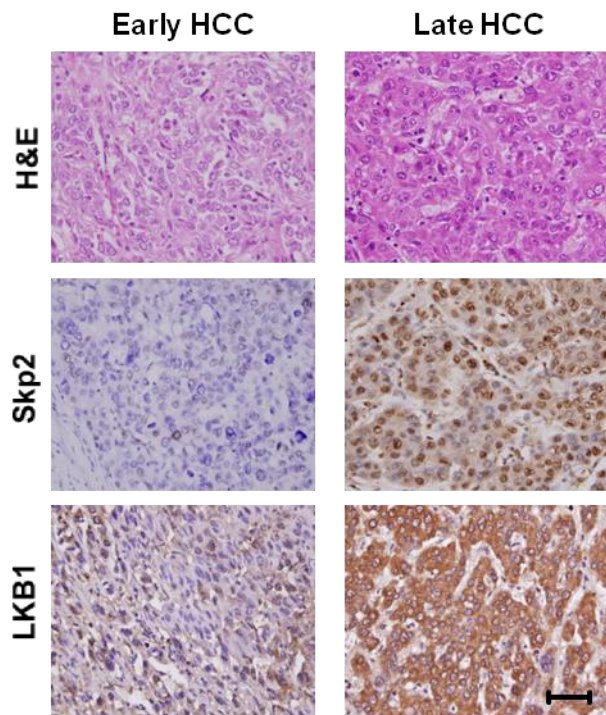
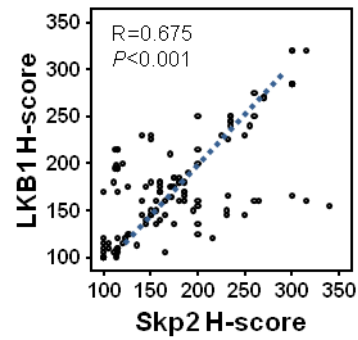
B**C**

Figure 3-28. Both LKB1 and Skp2 expression are upregulated and correlated in HCC (continued).

(B) Representative images of histological analysis of Skp2 and LKB1 staining in early (left panel) and late-stage HCC (right panel). Scale bar, 100 μ m.

(C) Scatter plot of Skp2 expression versus LKB1 expression in HCC samples.

The results **(B, C)** were carried out by two pathologists, Drs. Chien-Feng Li and Hsuan-Ying Huang.

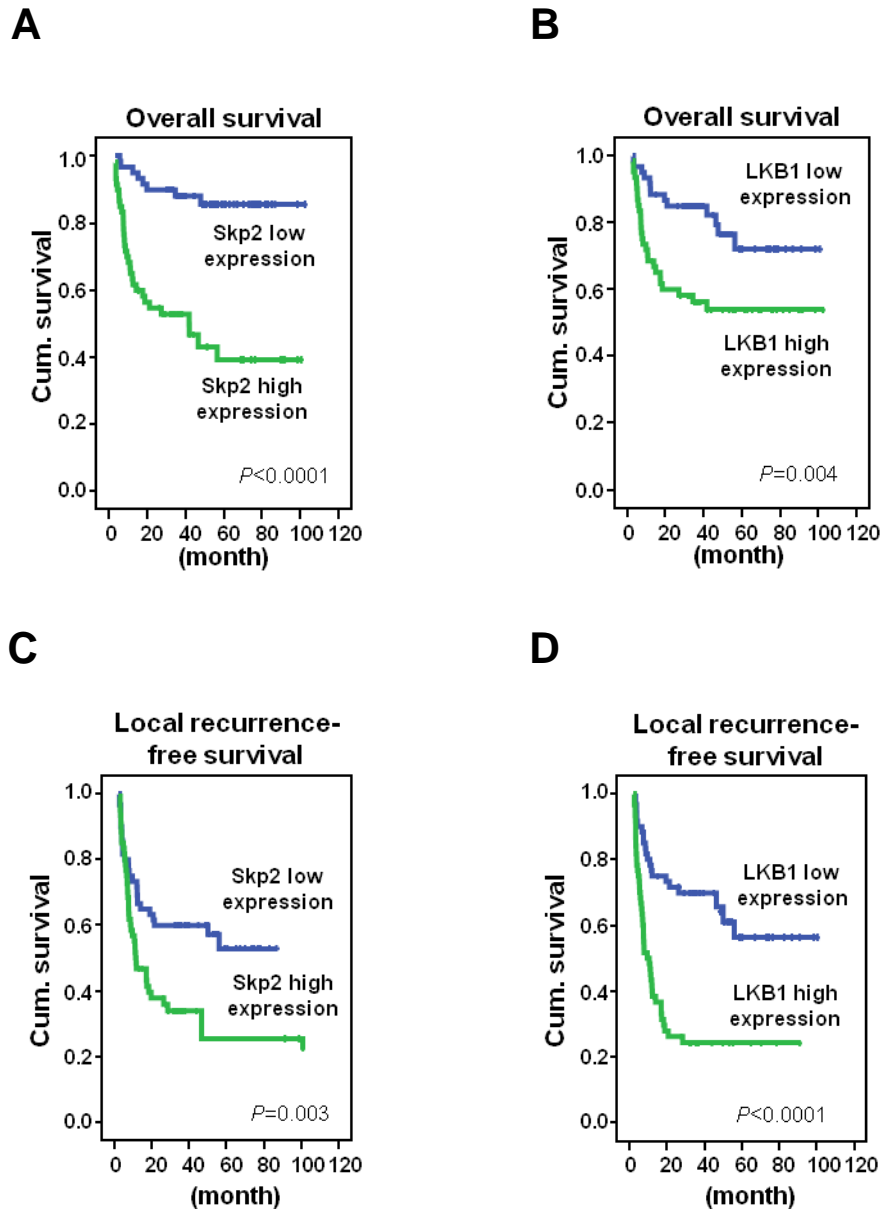


Figure 3-29. Overexpression of Skp2 or LKB1 predicts poor survival outcome of HCC patients.

(A) (B) Kaplan-Meier plots show that high expression of Skp2 **(A)** or LKB1 **(B)** is significantly predictive for inferior overall survival.

(C) (D) Kaplan-Meier plots show that high expression of Skp2 **(C)** or LKB1 **(D)** significantly predicts local recurrence-free survival.

The results **(A–D)** were carried out by two pathologists, Drs. Chien-Feng Li and Hsuan-Ying Huang.

Table 3-1. Correlations between Skp2 and LKB1 expressions to various clinicopathologic parameters

| | No. | Skp2 H-score | P-value | LKB1 H-score | P-value |
|------------------------------------|-----|-----------------|-------------------|----------------|-------------------|
| Sex | | | 0.063 | | 0.725 |
| Male | 93 | 169.18+/-56.94 | | 172.03+/-51.59 | |
| Female | 27 | 200.74+/-74.70 | | 183.41+/-66.55 | |
| Age (years) | | | 0.914 | | 0.882 |
| <60 | 53 | 172.06+/-49.43 | | 170.68+/-46.54 | |
| ≥60 | 67 | 179.63+/-71.29 | | 177.69+/-61.39 | |
| Hepatitis | | | 0.286 | | 0.619 |
| HBV | 57 | 167.28+/-53.11 | | 173.75+/-53.70 | |
| HCV | 47 | 181.15+/-69.95 | | 175.89+/-60.63 | |
| Both | 6 | 230.00+/-82.219 | | 200.00+/-48.89 | |
| None | 10 | 172.50+/-51.87 | | 158.00+/-38.74 | |
| Pugh-Child's Classification | | | <0.001* | | 0.005* |
| A | 109 | 167.28+/-56.75 | | 169.28+/-51.68 | |
| B | 8 | 290.00+/-186.51 | | 250.00+/-55.36 | |
| C | 3 | 200.00+/-0.00 | | 166.67+/-45.37 | |
| AFP | | | 0.028* | | 0.144 |
| <400 | 83 | 163.59+/-57.30 | | 165.34+/-50.78 | |
| ≥400 | 31 | 186.32+/-53.75 | | 181.87+/-52.13 | |
| Tumor Multiplicity | | | <0.001* | | <0.001* |
| Solitary | 68 | 153.26+/-43.43 | | 156.38+/-42.64 | |
| Multiple | 52 | 206.38+/-70.67 | | 198.40+/-60.86 | |
| Differentiation | | | 0.003* | | 0.031* |
| Well/Moderately differentiated | 93 | 166.94+/-58.22 | | 168.41+/-53.66 | |
| Poorly differentiated | 27 | 208.48+/-66.85 | | 195.89+/-56.18 | |
| Primary Tumor (pT) | | | 0.003* | | 0.083 |
| pT1 | 46 | 156.59+/-54.73 | | 163.52+/-55.99 | |
| pT2 | 39 | 174.05+/-59.92 | | 174.00+/-53.17 | |
| pT3-4 | 35 | 204.66+/-65.70 | | 189.80+/-54.37 | |
| AJCC Stage | | | <0.001* | | 0.012* |
| Stage I | 44 | 150.07+/-46.20 | | 156.41+/-45.72 | |
| Stage II | 39 | 174.05+/-59.92 | | 174.00+/-53.17 | |
| Stage III–V | 37 | 209.81+/-67.49 | | 196.84+/-60.69 | |
| CLIP score | | | 0.005* | | 0.097 |
| 0–1 | 85 | 166.27+/-59.25 | | 169.73+/-56.27 | |
| 2–3 | 35 | 200.60+/-64.20 | | 186.40+/-51.41 | |
| Okuda stage | | | 0.004* | | 0.076 |
| I | 85 | 163.80+/-53.43 | | 167.38+/-49.95 | |
| II–III | 35 | 206.60+/-72.56 | | 192.11+/-63.72 | |
| Skp2 Expression | | – | | r=0.675 | <0.001* |

*, statistically significant. The results were carried out by two pathologists, Drs. Chien-Feng Li and Hsuan-Ying Huang.

Table 3-2. Univariate survival analyses of HCC patients

| | | OS | | LRFS | |
|------------------------------------|-------------|--------------|----------|--------------|----------|
| Parameter | No. of Case | No. of Event | P-value | No. of Event | P-value |
| Sex | | | | | |
| Male | 93 | 29 | 0.5670 | 53 | 0.3622 |
| Female | 27 | 11 | | 14 | |
| Age (years) | | | | | |
| <60 | 53 | 16 | 0.4994 | 25 | 0.1622 |
| ≥60 | 67 | 24 | | 42 | |
| Pugh-Child's Classification | | | | | |
| A | 109 | 29 | <0.0001* | 56 | <0.0001* |
| B | 8 | 8 | | 8 | |
| C | 3 | 3 | | 3 | |
| AFP | | | | | |
| <400 | 83 | 20 | 0.0148* | 41 | 0.0820* |
| ≥400 | 31 | 14 | | 20 | |
| Tumor Multiplicity | | | | | |
| Solitary | 68 | 9 | <0.0001* | 26 | <0.0001* |
| Multiple | 52 | 31 | | 41 | |
| Differentiation | | | | | |
| Well/Moderately differentiated | 93 | 25 | 0.0010* | 47 | 0.0003* |
| Poorly differentiated | 27 | 15 | | 20 | |
| Primary Tumor (pT) | | | | | |
| pT1 | 46 | 7 | 0.0001* | 18 | 0.0003* |
| pT2 | 39 | 14 | | 24 | |
| pT3-4 | 35 | 19 | | 25 | |
| AJCC Stage | | | | | |
| Stage I | 44 | 5 | <0.0001* | 16 | 0.0001* |
| Stage II | 39 | 14 | | 24 | |
| Stage III–V | 37 | 21 | | 27 | |
| CLIP score | | | | | |
| 0–1 | 85 | 21 | 0.0001* | 41 | <0.0001* |
| 2–3 | 35 | 19 | | 26 | |
| Okuda stage | | | | | |
| I | 85 | 21 | 0.0002* | 44 | 0.0356* |
| II–III | 35 | 19 | | 23 | |
| Skp2 Expression | | | | | |
| Low expression (<median) | 60 | 8 | <0.0001* | 26 | 0.0030* |
| High expression (≥median) | 60 | 32 | | 41 | |
| LKB1 Expression | | | | | |
| Low expression (<median) | 60 | 13 | 0.0040* | 22 | <0.0001* |
| High expression (≥median) | 60 | 27 | | 45 | |

OS, overall survival; LRFS, local recurrence-free survival; *, statistically significant. The results were carried out by two pathologists, Drs. Chien-Feng Li and Hsuan-Ying Huang.

Table 3-3. Multivariate survival analyses of HCC patients

| Parameter | Category | OS | | | LRFS | | |
|------------------------|--------------------------------|-------|--------------|-------------------|-------|-------------|-------------------|
| | | RR | 95% CI | P-value | RR | 95% CI | P-value |
| Skp2 | Low expression (<median) | 1 | – | <0.001* | 1 | – | 0.120 |
| | High expression (≥median) | 7.059 | 3.044–16.367 | | 1.523 | 0.896–2.590 | |
| LKB1 | Low expression (<median) | 1 | – | 0.035* | 1 | – | <0.001* |
| | High expression (≥median) | 2.115 | 1.052–4.253 | | 3.027 | 1.774–5.164 | |
| CLIP score | 0–1 | 1 | – | 0.131 | 1 | – | 0.019* |
| | 2–3 | 2.134 | 0.798–5.708 | | 2.463 | 1.161–5.223 | |
| Okuda stage | I | 1 | – | 0.565 | 1 | – | 0.189 |
| | II–III | 1.288 | 0.544–3.049 | | 1.669 | 0.776–3.597 | |
| AJCC Stage | Stage I | 1 | – | 0.002* | 1 | – | 0.002* |
| | Stage II | 4.614 | 1.636–13.012 | | 2.500 | 1.297–4.820 | |
| | Stage III–V | 5.405 | 1.867–15.649 | | 2.971 | 1.427–6.184 | |
| Differentiation | Well/Moderately differentiated | 1 | – | 0.689 | 1 | – | 0.243 |
| | Poorly differentiated | 1.178 | 0.528–2.626 | | 1.243 | 0.776–2.728 | |

OS, overall survival; LRFS, local recurrence-free survival; *, statistically significant. The results were carried out by two pathologists, Drs. Chien-Feng Li and Hsuan-Ying Huang.

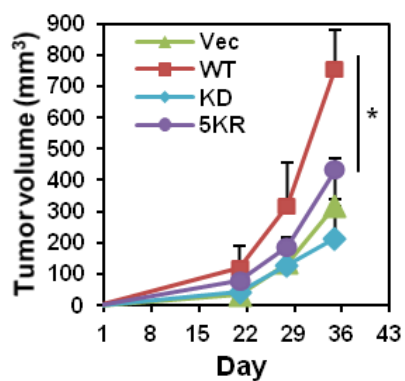


Figure 3-30. Overexpression of WT LKB1 promotes HCC tumor growth *in vivo*.

Hep3B cells with stable transduction of the indicated Flag-LKB1 were subcutaneously injected into nude mice. Tumor size was measured by the caliper, and the result is shown as means \pm s.d. ($n=3$). * $P<0.05$.

Chapter 4

Discussion

LKB1 has been suggested to be constitutively active (Sebbagh et al., 2011), and its activation is mediated by an allosteric mechanism dependent on complex assembly with STRAD and MO25 but is independent of the phosphorylation of the activation loop. All studies undertaken thus far have underlined the intrinsic interaction between each component within the complex for LKB1 activation (Boudeau et al., 2004; Milburn et al., 2004; Zeqiraj et al., 2009a; Zeqiraj et al., 2009b). However, how the LKB1 activity is maintained and whether the complex is regulated by other extrinsic proteins (e.g., other unidentified LKB1 regulators) remain unclear. In this study, we uncover that Skp2 is a novel regulator of LKB1 activation by promoting K63-linked polyubiquitination of LKB1. This posttranslational modification is important for LKB1 to bind to one of its subunits, MO25, but not the other, STRAD. Co-overexpression of LKB1 and STRAD enhances LKB1 activity by about 3-5 times, whereas the coexistence of MO25 in the LKB1-STRAD complex further boosts LKB1 activity by about additional 5-10 times (Boudeau et al., 2003; Zeqiraj et al., 2009b). Because the ubiquitination-deficient mutant of LKB1 identified in this study only compromises the LKB1-MO25 interaction but not the LKB1-STRAD interaction, the LKB1-5KR mutant still retains some kinase activity compared with the kinase-dead mutant of LKB1. To our knowledge, there have been no studies to date demonstrating LKB1 complex activation under any stimulating conditions (Sebbagh et al., 2011). Likewise, we did not observe that the physiologically relevant stimuli tested (e.g., glucose deprivation or growth factor treatment) could markedly induce more LKB1 polyubiquitination compared with the untreated controls (data not shown), and the defects of Skp2 loss and the LKB1-5KR mutant on LKB1 ubiquitination, complex integrity and activity could all be observed under the normal condition. These implicate that Skp2-mediated K63-linked polyubiquitination of LKB1 modulates the integrity and the activity of the LKB1 complex in a 'steady-state' manner regardless of

physiological stimuli.

The 5 K-to-R substitutions of the ubiquitination-deficient LKB1-5KR mutant are clustered at the N-terminus of LKB1 (Figure 3-13A). On the basis of the reported crystal structure of the LKB1-STRAD-MO25 complex (Zeqiraj et al., 2009a), these 5 Lys residues of LKB1 are not located within the key catalytic motifs. Therefore, the 5KR mutation supposedly would not affect the LKB1 kinase activity owing to amino acid substitution. In addition, the possibility that the 5KR mutation causes unanticipated structural alterations in LKB1 could be largely ruled out by the fact that the LKB1-5KR mutant binds to AMPK α , STRAD and Skp2 comparably to WT LKB1, exhibits the nucleocytoplasmic distribution similar to WT LKB1, and has the 'basal' kinase activity toward AMPK comparable to WT LKB1 when purified alone for the *in vitro* kinase assay (Figure 4-1). However, because the recombinant LKB1 protein Zeqiraj *et al.* used for crystallization is N- and C-terminus-truncated LKB1 protein, the importance of the 5 Lys residues of LKB1 in the intrinsic protein-protein interaction within the complex cannot be revisited by the reported crystal structure. Our results showing that polyubiquitination of LKB1 is not totally abolished on the 5KR mutant and upon Skp2 deficiency indicate that other LKB1 Lys residue(s) and other ubiquitin ligase(s) may be responsible for LKB1 ubiquitination. Because human LKB1 contains up to 26 highly conserved Lys residues, it is possible that ubiquitination of other Lys residue(s) on LKB1 by other ubiquitin ligase(s) contributes to regulation of LKB1 for activating different downstream signaling pathways. To test the assumption, screening for other potential ubiquitin ligase(s) toward LKB1 and mass spectrometric determination of LKB1 ubiquitination site(s) can be performed in the future.

K63-linked ubiquitination can regulate kinase activation, protein trafficking and protein-protein interaction mostly, if not all, without affecting protein degradation. K63-linked ubiquitin chains have been thought to adopt an extended open

conformation for interaction between ubiquitinated proteins and proteins containing ubiquitin-binding domains (UBDs) (Dikic et al., 2009). Our finding that K63-linked ubiquitination of LKB1 is critical for the LKB1-MO25 interaction highly suggests that MO25 might be an ubiquitin-binding protein that binds to the K63-linked ubiquitin chains of LKB1. MO25 is made up of 6 α -helical repeats with 3 helices and one with 2 helices (Milburn et al., 2004), forming a horseshoe shape binding both LKB1 and STRAD through its concave face (Milburn et al., 2004; Zeqiraj et al., 2009a). As most UBDs, if not all, bind to a hydrophobic patch of ubiquitin using α -helical structures (Dikic et al., 2009), the amphipathic nature of the MO25 helices with conserved hydrophobic patches within the helical repeats (Milburn et al., 2004) implicates the potential existence of unidentified UBD(s) in MO25. Hence, future investigations are needed to address the assumption and to determine how (i.e., the molecular mechanism) Skp2-mediated K63-linked polyubiquitination of LKB1 modulates the LKB1-MO25 interaction. To this end, the *in vitro* binding assays by which the interaction between recombinant GST-MO25 and K63-linked tetra-ubiquitin will be assessed. Furthermore, the minimal binding region of MO25 to polyubiquitin chains will be mapped by using various truncated and/or deleted mutants of MO25 in the *in vitro* binding assays.

In this study, we show that oncogenic Ras is an upstream regulator of Skp2 and LKB1, which activates the Skp2/LKB1/AMPK axis to maintain cancer cell survival under metabolic stress by promoting Skp2-dependent LKB1 polyubiquitination (Figure 4-2). However, the molecular mechanism of how the Ras signaling cascade promotes the Skp2-SCF activity toward LKB1 remains to be defined. The Ras-mediated effect may work on either LKB1 or Skp2, or both. Previously, the Ras/Raf/MEK/Erk signaling has been shown to impact LKB1 function via Erk-mediated Ser325 and p90Rsk-mediated Ser428 phosphorylation of LKB1 (Esteve-Puig et al., 2009;

Martinez-Lopez et al., 2012; Zheng et al., 2009) (*Introduction* 1.5). In our case, phosphorylation of LKB1 at either site or both did not consistently affect Skp2-mediated LKB1 ubiquitination (data not shown). It has been thought that the F-box protein subunit of SCFs specifically recognizes and binds their substrates that undergo phosphorylation (Frescas and Pagano, 2008). One study demonstrated that active Erk-directed phosphorylation of the Skp2 substrate MKP1 (MAPK phosphatase 1; also known as DUSP1) (Table 1-4) promotes MKP1 binding to the Skp2-SCF and subsequent MKP1 ubiquitination (Lin and Yang, 2006). Therefore, it is likely that other LKB1 phosphorylation site(s) that can be modified by Ras downstream kinase(s) have not been identified, which is suggested by the computational prediction of LKB1 phosphorylation sites. The phospho-site prediction of human Skp2 indicates that there are three potential phosphorylation sites for Raf kinase, some for MEK (also known as MAPKK), and one or two for MAP kinases (Erk, Jnk and p38). Future experiments will be needed to address whether or not the predicted LKB1 or Skp2 phosphorylation occurs and affects the Ras/Skp2/LKB1 axis.

Deregulated activation of Ras signaling is frequently found in human HCC. Given that Ras-driven tumors are highly aggressive and likely experiencing severe metabolic stress inside the tumors, it is therefore conceivable that in order for Ras-driven tumors to develop into a full-blown disease, the cancer cells must activate a survival program (e.g., the Skp2/LKB1/AMPK axis shown here) to counteract metabolic stress-induced cell death. A recent study has underscored the significance of a functional LKB1/AMPK pathway in cancer cell survival in response to metabolic stress (Shackelford et al., 2013). Shackelford *et al.* showed that in a mouse model, non-small cell lung cancers which lack a functional LKB1/AMPK pathway are more sensitive to the treatment with the metabolic drug phenformin, which inhibits the mitochondrial complex I in the electron transport chain and results in the elevation of

intracellular AMP levels. In support of their notion, we found that LKB1 knockdown sensitized Hep3B HCC cells to phenformin (Figure 4-3A). Moreover, inactivation of Skp2 either genetically by Skp2 knockdown or pharmacologically by the Skp2 inhibitor, which both downregulated the LKB1/AMPK signaling, enhanced the sensitivity of Hep3B HCC cells to phenformin treatment (Figures 4-3B,C). Therefore, we reasoned that a combination therapy with phenformin and the Skp2 inhibitor may be an effective strategy for treating certain types of cancers, e.g., HCC.

Treating Ras-driven cancers is a challenging task due to the extreme complexity of the Ras signaling networks, which thereby leads to unwanted therapeutic outcomes, such as drug resistance, and off-target effects (Stephen et al., 2014). Our findings that Ras activates the Skp2/LKB1 pathway to promote cancer cell survival under energy stress may shed light on potential therapeutic implications for targeting a new branch of the Ras signaling networks (i.e., the Ras/Skp2/LKB1 axis). To further test this notion, we treated Ras-overexpressing cancer cells with the Skp2 inhibitor, which interrupts the Ras/Skp2/LKB1 cascade similar to the knockdown of Skp2 or LKB1 and the LKB1-5KR mutant. We found that Ras-overexpressing Hep3B cells were very sensitive to Skp2 inhibitor treatment in a dose-dependent manner (Figure 4-4A). Moreover, administration of the Skp2 inhibitor further sensitized the Ras-overexpressing cells to metabolic drug phenformin (Figure 4-4B). Our data therefore provide a proof-of-principle that the combination treatment with the Skp2 inhibitor and metabolic drugs may be effective for treating Ras-driven cancers.

Our findings that LKB1 is oncogenic in HCC appear paradoxical because Lkb1 has been shown to display the tumor-suppressive activity in various genetic mouse models (Ollila and Makela, 2011; Shorning and Clarke, 2011) (Table 1-1). In this study, we propose that during tumor development, activation of the Ras/Skp2/LKB1 axis is required for cancer cell survival during energy stress. Therefore, our results are in line

with the currently accepted understanding of LKB1 function in stress-induced cell survival (Shaw et al., 2004b). Most *Lkb1* genetic mouse models reported to date used tissue-specific knockout strategies (Tables 1-1 and 1-2); however, knockouts with chronic inactivation of *Lkb1* could not address the temporal requirement of Lkb1 throughout the course of cancer development, especially at the later stage where severe metabolic stress takes place within the tumor microenvironment. Thus, the role of LKB1 in cancer progression and maintenance is still largely undefined at present. Our result showing the enhanced expression of LKB1 and Skp2 in late-stage HCC implicates the oncogenic function of LKB1 in liver cancer progression rather than initiation. By contrast, we observed the tumor-suppressive activity of LKB1 in subcutaneously injected A549 lung tumor xenografts in agreement with other reports (Figure 4-5). Accordingly, generation of appropriate mouse models with conditionally manipulatable (i.e., temporally and spatially inducible) knockout or knockin of *Lkb1* will be absolutely required to answer the paradoxical, complex roles (i.e., the oncogenic, tumor-suppressive, context- and tissue-specific roles) of LKB1 in tumorigenesis. A simple alternative can be applied using the Tet-On shRNA system and the Tet-On/Off inducible vector in different types of cancer cells for temporally inducible knockdown and expression of LKB1 in mouse orthotopic xenograft (or allograft) tumor models.

In addition to AMPK, LKB1 is capable of phosphorylating and activating 12 AMPK-related kinases (ARKs), thereby directing multiple cellular processes (Bright et al., 2009; Lizcano et al., 2004) (Figure 1-1 and Table 1-3). Given that our data show that LKB1 ubiquitination modulates LKB1 complex integrity and activity, it is speculated that Skp2-mediated K63-linked ubiquitination of LKB1 may also affect other LKB1 downstream effectors besides AMPK for regulation of energy stress responses or other functions. Generally, microtubule affinity-regulating kinases

(MARK1–4) are primarily involved in cell polarization, and brain-specific kinases (BRSK1 and BRSK2), expressed almost exclusively in the brain, mainly regulate neuronal polarity and axon specification. Salt-inducible kinases (SIK1, SIK2 and SIK3) have different functions from one another (Table 1-3) likely due to their different tissue-specific expression, and SIK1 has been shown to regulate p53-dependent anoikis (Cheng et al., 2009). Among those LKB1 downstream substrates, the novel (nua) kinase family (NUAK) NUAK1 (also known as ARK5; AMPK-related kinase 5) and NUAK2 (also known as SNARK; Snf1/AMPK-related kinase) are of our interest because they have been suggested to be involved in cancer cell survival, migration and metastasis, and pro-oncogenic (Sun et al., 2013) (Table 1-3). Liu *et al.* showed that NUAK1 is required for Myc-driven cancer cell survival *in vitro* by maintaining cellular energy homeostasis and for tumorigenesis in a orthotopic mouse model of HCC (Liu et al., 2012b), and elevated NUAK1 expression was observed in several types of human cancers, including HCC (Cui et al., 2013; Liu et al., 2012b). Also, NUAK1 has been reported to mediate Akt-dependent cancer cell survival and migration (Suzuki et al., 2004) and suppress cell apoptosis under energy stress (Suzuki et al., 2003a). SNARK appears to be activated in response to AMP (Lefebvre and Rosen, 2005) and plays a role in cancer cell motility (Suzuki et al., 2003b). As we observed that LKB1 promoted both the *in vivo* HCC tumor growth in a mouse subcutaneous xenograft tumor model (Figure 3-30) and the *in vitro* HCC cell migration (Figures 4-6A,B) in a kinase activity- and ubiquitination-dependent manner, we reason that LKB1 may execute its pro-oncogenic, ubiquitination-dependent function in HCC via its pro-oncogenic downstream substrate(s), i.e., NUAK1 and/or NUAK2. Therefore, the effect of Skp2-mediated LKB1 polyubiquitination on the LKB1/NUAK axis will be investigated, and the expression correlation of LKB1 with NUAK1 and NUAK2 will also be assessed in our clinical HCC samples. Because different LKB1 binding affinity

to its different ARK substrates has been observed (Lizcano et al., 2004), we will further determine whether Skp2-mediated K63-linked ubiquitination of LKB1 contributes to LKB1 substrate-binding specificity.

In summary, our study shows that Skp2-mediated K63-linked polyubiquitination of LKB1 is critical for maintaining the integrity of the LKB1-STRAD-MO25 complex, thereby modulating LKB1 activity and cellular function in stress-induced cell survival responses, and that Ras acts upstream of this Skp2/LKB1 pathway (Figure 4-2). Identification of the Ras/Skp2/LKB1 axis in this study not only provides great insight into how LKB1 kinase activity is maintained, but also offers new therapeutic strategies for targeting Ras-driven cancers.

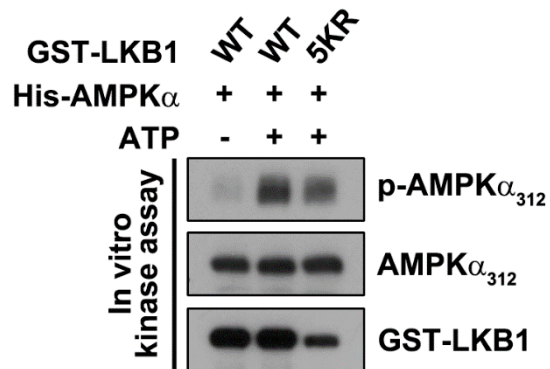


Figure 4-1. The 5KR mutation does not impact the kinase activity of recombinant GST-LKB1.

The indicated recombinant GST-LKB1 proteins were purified from bacteria and subjected to *in vitro* LKB1 kinase assay followed by immunoblotting. Recombinant His-AMPK α_{1-312} protein was purified from bacteria and used as the substrate.

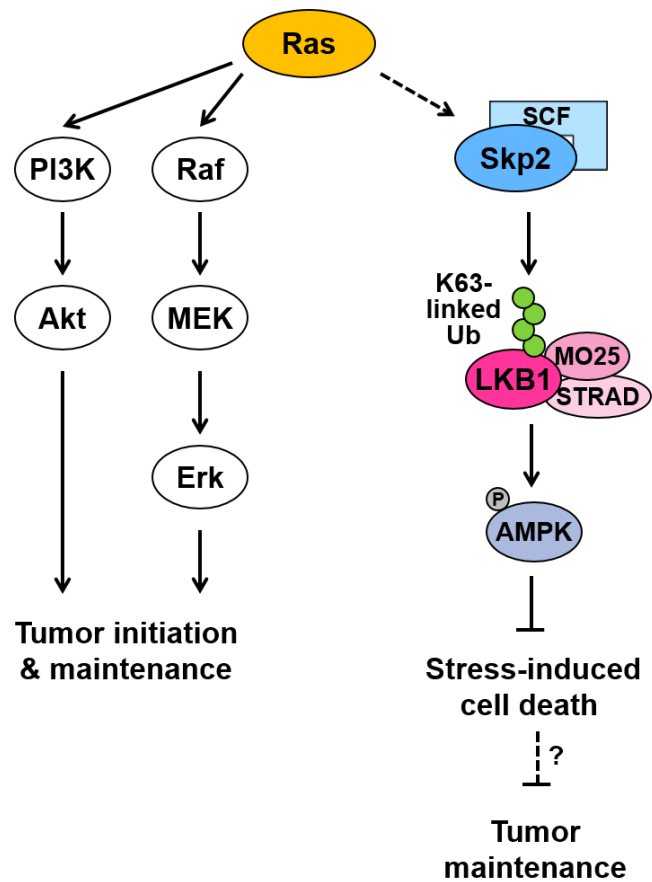


Figure 4-2. Schematic representation of the working model of this study.

The Ras/Skp2/LKB1 pathway promotes cancer cell survival during energy stress and may play a role in tumor maintenance. However, future investigation is needed to prove the role of the Ras/Skp2/LKB1 axis in tumor development, which is indicated by a question mark. Ub, ubiquitination; P, phosphorylation.

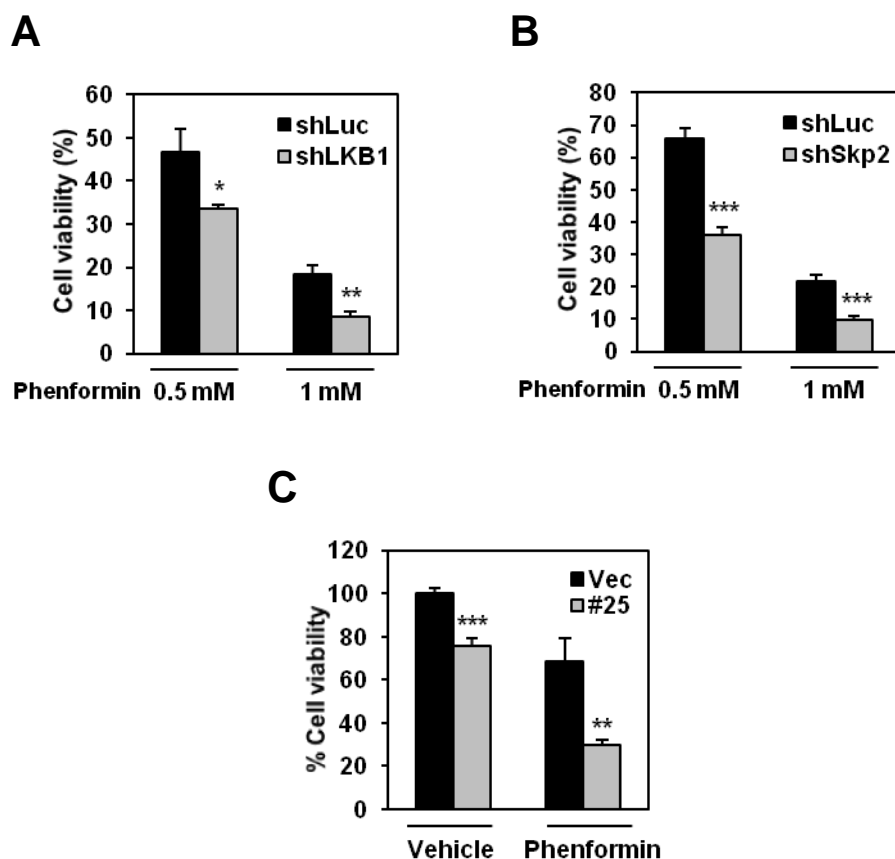


Figure 4-3. Targeting LKB1 or Skp2 sensitizes HCC cells to phenformin treatment.

(A) (B) Cell viability of Hep3B cells with control (shLuc), LKB1 **(A)** or Skp2 knockdown **(B)** after treatment with phenformin at the indicated concentrations for 3 days was determined by trypan blue exclusion assay, and is expressed as a percentage of the untreated controls.

(C) Cell viability of Hep3B cells after combined treatment with Skp2 inhibitor (compound #25) and phenformin for 3 days was determined by trypan blue exclusion assay, and is expressed as a percentage of the vehicle-treated controls.

All results are shown as means \pm s.d. ($n=3$). * $P<0.05$; ** $P<0.01$; *** $P<0.005$.

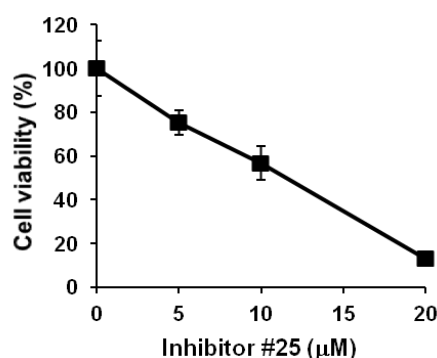
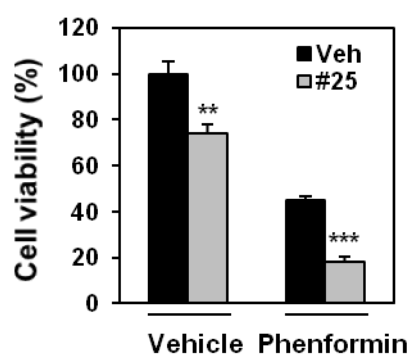
A**B**

Figure 4-4. The Skp2 inhibitor displays a potent inhibitory effect on HCC cells with Ras hyperactivation.

(A) Cell viability of Hep3B cells with stable transduction of Ras after treatment with the Skp2 inhibitor (compound #25) at the indicated concentrations for 3 days was determined by trypan blue exclusion assay, and is expressed as a percentage of the vehicle-treated control.

(B) Cell viability of Hep3B cells with stable transduction of Ras after combined treatment with Skp2 inhibitor (compound #25) and phenformin for 3 days was determined by trypan blue exclusion assay, and is expressed as a percentage of the vehicle-treated controls.

Both results are shown as means \pm s.d. ($n=3$). ** $P<0.01$; *** $P<0.005$.

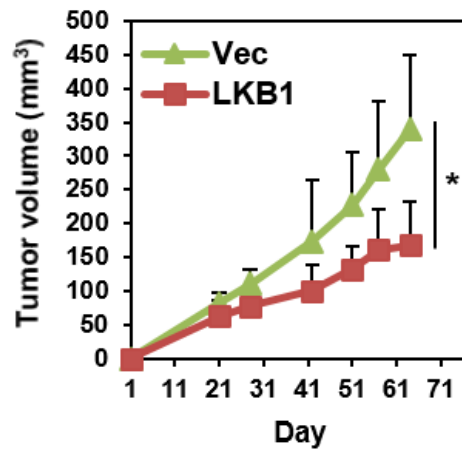
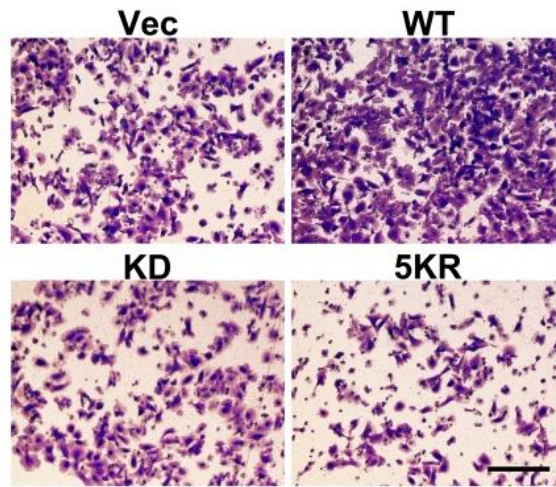


Figure 4-5. Overexpression of WT LKB1 attenuates lung cancer cell growth *in vivo*.

1.5x10⁶ A549 lung cancer cells with stable transduction of vector (Vec) or Flag-LKB1 were subcutaneously injected into nude mice. Tumor size was measured by the caliper, and the result is shown as means \pm s.d. ($n=4$). * $P<0.05$.

A



B

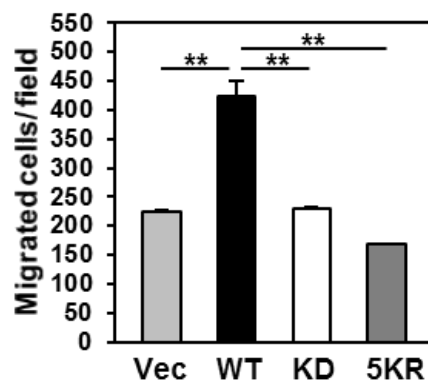


Figure 4-6. Overexpression of WT LKB1 promotes HCC cell migration.

(A) (B) Transwell migration assay in which Hep3B cells with stable transduction of vector (Vec) or the indicated Flag-LKB1 were inoculated in serum-free medium into the upper chamber of the well. After 24 hours, migrated cells were fixed, stained with crystal violet and observed under the microscope. Representative images **(A)** and the quantitative result **(B)** are shown. The result is shown as means \pm s.d. ($n=3$). ** $P<0.01$. Scale bar, 200 μ m.

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